"STUDIES ON THE PASSIVE SENSITIZATION OF HUMAN LUNG".

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SUMMARY

Study concerns passive sensitization of human lung with human reaginic antibody, and experiments to illustrate similarities and differences between human and guinea-pig lung tissue during anaphylaxis.

Conditions suitable for in vitro sensitization of chopped human lung have been investigated. Soaking at 17°C for at least 15 hours in Tyrode solution containing selected reaginic serum, regularly produces sensitization. Serum from patients sensitive to the pollen of cocksfoot (Dactylus glomerata) have been used routinely, but not exclusively.

The level of sensitivity has been assessed by the percentage of tissue histamine which is released on challenge with an aqueous extract of pollen antigen. The lung parenchyma has been shown to be satisfactory in use and provides large amounts of uniform tissue which may be divided in triplicate samples. Tissue chopped into rods about 0.8 x 0.8 mm is satisfactory and convenient to use. When previously sensitized tissue was incubated at 37°C with reaginic serum for up to 2 hours, sensitization declined. When the tissue was

incubated at 37°C after long periods at 17°C there was a sharp increase in the rate of spontaneous histamine release. Attempts to reduce this by the use of different bathing fluids were not successful. The degree of sensitization as judged by the histamine release is roughly proportional to the concentration of reaginic serum in which the tissue has been sensitized. Sensitization is enhanced if the γ_2 globulins are removed from the serum and is diminished if γ_2 globulin (which is not antibody to pollen) is added to the sensitizing serum. This shows that there must be competition for uptake of antibody globulins on the tissue.

A wide sweep of sera collected from patients presenting for hayfever and asthma, and all showing strong positive skin reactions to mixed grass pollen extracts, showed that there was no correspondence between the production of skin tests and the power of the serum to sensitize the lung. Usually the sensitizing property of the serum was not changed as a result of 6-12 months treatment with gluco-corticoids, but it has been markedly diminished by a course of hyposensitiz-

ation therapy, in a single case. The use of tissue removed at operation introduces a major variable into the experiments; generally speaking, healthy looking tissue with thin smooth pleura is most satisfactory, and brownish tissue is not. The content of soot and the presence of a thick opaque pleura does not necessarily indicate an unsatisfactory tissue but the histamine content is often low. The retention of histamine may be taken as an indication that the tissue is satisfactory.

Human lung shows many features already established in guinea-pig lung, when challenged in vitro. These include requirement of Ca^{++}, pH around 7.6, temperature 37°C-38°C and desensitization by exposure to 45°C for 20 minutes. Correspondence was also good in respect of the inhibition by substances acting against chymotrypsin and in respect of enzymes dependent upon the integrity of S-H groups. Salicylaldoxime also blocks very effectively.

Differences from the guinea-pig lung were seen in the failure of succinic acid and of caproic acid to alter the reaction in human tissue. A considerable amount of slow reacting substance (SRS-A) was evident in some experiments.

Separation of different protein constituents of the pollen extract have indicated that allergic patients show variation in the substances to which they are sensitive. This would greatly complicate experiments using radio-labelled antigen, and for such studies a simpler antigen antibody system would be preferable. Identification of the antigen to which a given patient is principally sensitive is possible by the in vitro technique now developed; and could lead to the use of safer and more reliable antigens in clinical skin and provocation tests, and particularly in hyposensitization.

INTRODUCTION

Hypersensitivity

The word allergy is derived from the Greek and means altered energy or altered reactivity. The existence of allergy and the hypersensitive state in the human being has long been known. The allergic individual reacts differently to certain substances with which he comes in contact; what is harmless for non-allergic people causes a variety of unpleasant reactions in allergic people. The range of substances which can produce allergic disorders is very wide, odour of flowers, dust from animals and plants, stings from plants and insects, many chemicals and food stuffs, and, in exceptional cases, even products from other humans.

Anaphylaxis

The first experimental demonstration of the hypersensitive state was made in 1902, when Portier and Ritchet introduced the term anaphylaxis, to describe the characteristic and violent reactions which they had observed in dogs, on re-injection of substances which were not necessarily toxic. The modern concept of the development of this altered state can be based on the report of Von

Pirquet and Schick (1905) concerning serum sickness. These authors attributed the clinical manifestations following repeated injections of heterologous serum, to an antigen-antibody reaction in vivo. They explained that horse or rabbit serum used to give passive immunity in humans, acts as an antigen and leads to the formation of antibody. The time taken for this production of antibody was called the incubation period, and injection of the antigen during this period would not produce the anaphylactic reaction. Variations in the type of reaction to antibody (immune heterologous serum) injected after the incubation period were attributed to the presence of different amounts of antibody.

In 1907, it was demonstrated by Otto and by Friedman in guineapigs, Nicolle in rabbits, and Ritchet in dogs that when the serum from a sensitized animal was injected into a normal animal, anaphylaxis could be produced after 2 to 3 days. It was therefore clear that the specific antibody was fundamental in the development of hypersensitivity. In 1913, Weil showed that the severity of anaphylactic shock in actively sensitised guineapigs increased as the amount of precipitating

antibodies in the circulation fell. This indicated that the antibody was being fixed or transferred to the tissues. The need for a considerable latent period between the injection of the antibody and the development of hypersensitivity, indicated that reactions in circulating blood did not produce full anaphylactic shock. Studies in vitro with washed organs (Dale 1912) showed that the antibody had in some way to modify tissue cells, before hypersensitivity was demonstrable. In 1921, Prausnitz and Kustner published the results of their observations on passive transfer of local cutaneous hypersensitivity to non-sensitive humans by intradermal injection of the serum from a sensitive person. This work showed that local antigen-antibody reactions in man also needed a period for the "fixation" of antibody onto tissue, and that as in animals, hypersensitivity was not dependent upon any abnormality of the tissue.

It is now well established that the union of antigen and antibody in the anaphylactic reaction leads to the release of autopharmacologic substances both in vitro and in vivo. Histamine may not always be the most

important of these, but is usually involved and relatively easy to detect. There have always been workers who believed that the tissues were the principal site of antigen - antibody reactions, and others who thought the reaction in blood was more important. Each side had evidence.

Dragstedt, and Gebauer-Fuelnegg in 1932 reported that a considerable amount of histamine was released from the liver into the thoracic duct lymph of sensitised dogs, when the antigen was injected. Code in 1939 showed a striking release of histamine in the blood of sensitised guineapig when challenged with the antigen. Addition of antigen to heparinised whole blood of sensitised rabbits (Katz 1940), results in considerable release of histamine from the white cells into the plasma. According to Humphrey and Jacques (1955) much of the histamine thus liberated is derived from the blood platelets, which in rabbits are particularly rich in histamine content. Katz and Cohen in 1941 reported in vitro antigen-induced histamine release from blood of hay fever sensitive patients, while Noah and Brand in

1954 showed the release of histamine from the white blood cells from a number of sensitive cases, using a wide variety of allergens. Those who considered that tissue was the more important site of the antigen - antibody reactions, laid stress on reactions which occurred in vitro, or in the absence of blood.

Bartosch, Feldberg and Nagel in 1932, demonstrated the presence of histamine in the effluent, when the lungs of a sensitized guinea pig, were perfused free of blood with Ringer solution, and then challenged with the specific antigen added to the perfusion fluid.

In 1937, Schild showed that various isolated organs from actively sensitized guinea-pigs, washed to remove blood, released histamine when challenged with the antigen, at 37°C. He also confirmed the work of Bartosch et al, and obtained better yields of histamine. In all the above work histamine was identified by biological assay in the presence of atropine.

Dale (1912) demonstrated that uteri from passively sensitized guinea pigs, washed free of blood, showed strong and immediate contraction, when the appropriate

antigen was added to the bath. Once a maximal response had been obtained by the addition of the antigen, the tissue became desensitized to further doses of the same antigen. The tissue could however be resensitized by soaking for several hours in the sensitizing serum.

Hartley in 1939 demonstrated the importance of species compatibility. He reported that uteri from normal guinea-pigs could be passively sensitized in vitro, by soaking overnight in cold impotent antiserum from guinea-pig, rabbit, or man, but not by antiserum from goat, sheep, ox, or horse.

Benacerraf and Kabat (1949) used accurately assayed antibody in quantitative studies on the development of sensitization in vivo in guinea-pigs. The latent period between the administration of the antibody and the development of passive sensitization, as shown by anaphylactic shock, was inversely proportional to the amount of antibody injected. They concluded that the amount of antibody fixed determined the severity of shock.

Studies in vivo are necessarily cumbersome due to animal variation, and are either all or none, or involve

the subjective evaluation of intensity of shock. They are hard to interpret because several interrelated mechanisms may be involved, furthermore many tests (e.g. with metabolic inhibitors) are not possible. The work of Dale and Hartley, showed the practicability of passive sensitization and in vitro anaphylactic reaction with whole organs.

The evaluation of anaphylactic reactions in vitro by measuring the amount of histamine set free from fragments or slices of lung tissue was developed by Mongar and Schild in 1953. When antigen was added to a suspension of minced lung from a sensitized guinea-pig up to 40% of the total histamine was shown to be released from the cells into the supernatant fluid. In 1957, these authors extended this principle to passive sensitization of chopped guinea-pig lung, by suspending the fragments of tissue in antiserum diluted with Tyrode solution. A significant release of histamine would occur on challenge, only when the tissue had undergone at least one hour's incubation at 37°C, with the anti-serum. Studies on the superficial biochemistry of the

anaphylactic reaction in vitro, showed that the amount of histamine released during anaphylaxis, was affected by temperature. Both high and low temperatures were found to reduce the anaphylactic histamine release, which was maximal at 38-40°C. Other conditions necessary during challenge by antigen were the presence of calcium ions in the suspending solution, and pH around 7.5-8. This indicates that the anaphylactic histamine-releasing process involves the activation of an enzyme.

Halpern and colleagues in 1959, soaked guineapig ileum in diluted rabbit antiserum and evaluated the sensitization by the histamine equivalent of the Schultz-Dale reaction produced subsequently. They showed that the degree of sensitization was proportional to the concentration of the antibody used to sensitize the tissue, and that the time to reach a given degree of sensitization was disproportionately decreased by raising the concentration of the antibody *ie.* $[Ab] \times t^2 = k$, over a range of temperature 10-38°C. These authors also reported that the rate of sensitization was not affected by pH over the range 8.2 to 5.6. But showed by the use of

bicarbonate buffers, that high levels of CO_2 prevent sensitization of the tissue, so that pH in vivo will have an effect.

Humphrey and Mota (1959) studying the in vitro uptake of radio-labelled antibody onto the guinea-pig mesentery found that rates of uptake of rat, rabbit, and horse antibody were substantially the same, regardless of whether the tissue was at 37°C or 0°C, and whether metabolic inhibitors were present or not, in spite of the fact that only rabbit γ-globulin was capable of producing sensitization.

Brocklehurst, Humphrey and Perry (1961) used pre-perfused chopped guinea-pig lung, and partially purified radio-labelled antibody fractions from highly immunised rabbits to measure the adsorption of antibody during passive sensitization in vitro. They demonstrated that antibody rapidly became attached to the tissue. With a very high concentration of antibody at 4°C detectable sensitization occurred within 30 seconds, and an almost maximal degree of sensitization in about 30 minutes.

It was also shown that the amount of antibody taken up by the tissue was proportional to the concentration

of the antibody in the surrounding solution. These authors demonstrated that rates of adsorption of antibody were different at 37°C, and at 4°C. For the same antibody concentration, and same length of time, the antibody uptake at 37°C was twice that at 4°C, and the sensitization as judged by anaphylactic histamine release at 37°C was more than double that of 4°C. Brocklehurst and Colquhoun (1965) have used ^{131}I-labelled γ_1 guinea-pig antibody and labelled total rabbit γ-globulin to show that the uptake of the globulin by guinea-pig lung is directly proportional to the applied concentration over a very wide range. They also showed that there was no simple relationship between the amount of γ-globulin taken up and the level of sensitization produced, and further that sensitization increased with prolonged contact although there was no measurable increase in the amount of antibody adsorbed. Feigen et al (1962) have studied the uptake of radio-labelled rabbit anti-ovalbumin, using strips of guinea-pig ileum, over the temperature range 20-37°C, and a range of antibody concentrations. They have shown that there was no essential difference in the rates of adsorption of

γ-globulins, over this temperature range, but confirmed that uptake was directly related to the applied concentration of the antibody.

The value of all the above observations, regarding antibody uptake is now doubtful, because the antibody used must in practically every instance have been a mixture of the different types of immune globulins, and species variations in antibody were scarcely recognised.

The choice of a suitable tissue will have a very strong bearing on the validity and the possible range of tests, and is therefore very important. The tissues used seem all too often to be far from ideal and chosen for apparent simplicity or personal familiarity. Halpern and colleagues (1959), and Feigen and associates (1962), used isolated guinea-pig ileum to assess sensitization by the magnitude of the Schultz-Dale response. The use of an intact organ has several disadvantages. The membranes (serosal and mucosal) will influence the uptake of antibody and the rate of access of antigen. Even adjacent samples from the same tissue are not exactly comparable, as their response to histamine

varies a great deal, and their ability to produce a response to other mediators, cannot be tested. Anaphylactic contraction of the gut is attributed to multiple agents released from within, and therefore comparison with contraction caused by externally applied histamine is not valid, (Reuse, 1956). Moreover the measured response is only an indirect outcome of sensitization, the total number of preparations (tissue-samples) is very limited, and that the reactions are obtained at different times during the investigations.

Before 1965, no successful attempts had been made to passively sensitize isolated human tissues in vitro. The Prausnitz-Küstner test was the only means routinely available to detect reaginic antibodies in sera from allergic persons, and it was widely held by clinicians that reagins were quite unlike experimentally produced antibodies, (Payling-Wright 1957).

There was nevertheless a body of evidence showing that tissues from allergic individuals had a great deal in common with guinea-pig tissues from sensitized animals when subjected to challenge in vitro. Schild, Hawkins, Mongar and Herxheimer in 1951, reported studies with

chains of human bronchioles, and fragments of lung. Samples of lung obtained postoperatively from a pollen sensitive patient, were cut into small pieces and placed in Tyrode's solution at 37°C. When challenged with the antigen, the tissue released histamine into the surrounding fluid. It was found that a chain of bronchial rings from the same lung, contracted strongly when the pollen antigen was added to the bath and released histamine at the same time. The bronchial muscle was unresponsive to the subsequent addition of the same pollen, although its responses to histamine and acetylcholine remained unaffected. Similar tissues from non-allergic patients did not release any histamine. It was shown that even high concentrations of mepyramine (4µg/ml) failed to abolish the contraction of the bronchial chain on adding the antigen. Since no other active substances were detected; this effect was attributed to intrinsic histamine. However, Schild in 1956, stated that this effect could be due to the release of other plain muscle stimulants, which were not affected by mepyramine. In 1956, Brocklehurst clearly showed that both histamine and SRS-A, were

present in the perfusate, when the specific antigen had been added to the perfused lungs of sensitized guinea-pigs, and of a pollen sensitive patient. Chopped lung from 3 other asthmatic cases released histamine and SRS-A in vitro on coming into contact with the appropriate allergen. (Brocklehurst and Mongar - quoted in Brocklehurst 1960). Thus there appeared to be sufficient evidence that lung tissue from sensitized guinea-pigs and from asthmatic humans react similarly on challenge in vitro; but wider study of the human tissues was desirable to demonstrate in detail the closeness of correspondence.

Tissues from adequately documented asthmatic patients are very seldom available, and in view of the extensive studies on passive sensitization of guinea-pig tissues, the in vitro sensitization of human tissue seemed quite feasible.

The nature of antibody involved in sensitizing guineapig tissues:

Ovary, Benacerraf and Bloch (1963) observed fatal systemic anaphylaxis in normal guinea-pigs when a purified γ_1 antihapten fraction was injected intravenously and challenged with the antigen by the same

route after a latent period of 40 hours. No signs of anaphylaxis were seen in guinea-pigs similarly treated with γ_2 fraction.

Baker, Bloch and Austen in 1964, showed that only γ_1 anti-hapten antibody and purified γ_1 globulin from immune guinea-pig sera were capable of sensitizing chopped and thoroughly washed normal guinea-pig lung tissue in vitro.

Brocklehurst and Colquhoun (1965), reported essentially similar results with homologous anti-ovalbumin and anti-bovine serum albumin γ-globulin fractionated by methods unlikely to produce changes in the configuration of the antibody.

Ovary and Bier (1953) were the first to show that other γ-globulins could interfere with the sensitization produced by a specific antiserum. They showed that for minimal passive cutaneous sensitivity in the guinea-pig, antibody equivalent to 0.01 µg. N. was required if sensitizing rabbit antiserum was diluted in saline, but if the immune serum was diluted in normal rabbit serum 50 times more antibody was needed to produce the same effect, and 100 times as much needed, if it was diluted

in rabbit γ-globulins. These authors also showed that a latent period between sensitization and injection of antigen was required before PCA could be elicited, and that the latent period was reduced by increasing the sensitizing dose of antibody. In 1962 Ovary and Benacerraf showed that the guinea-pig antisera against haptenic antigens contained two populations of precipitating antibody molecules, and that only the electrophoretically fast component, identified as γ_1 globulin could sensitize guinea-pigs for systemic or passive cutaneous anaphylaxis. White, Jenkins and Wilkinson (1963), reported similar results. Later, Ovary, Benacerraf and Bloch (1963), showed that the PCA caused by homologous γ_1 antibodies could be blocked by excess γ_2 antibodies to the same antigen, presumably by competition for the antigen.

Colquhoun and Brocklehurst (1965), showed that in vitro sensitization of guinea-pig lung could be achieved with very low amounts of γ_1 antibody, whereas γ_2 antibodies in concentrations 400 times greater showed only slight activity. However, they produced comparable PCA reactions with amounts of γ_2 antibody only 30 times

greater than those of γ_1, provided that intravenous challenge was not delayed more than 6 hours after the intradermal doses of antibody. A difference in sensitizing potency comparable to that found when chopped lung was used, was seen with the PCA test when the challenging antigen was injected about 2 days after the antibody. The γ_1 antibody was then several hundred times more effective than γ_2. The γ_1 antibody is evidently much more firmly held at the injection site than is γ_2. In traditional P-K tests, the antibody site is challenged locally after a period of about 24 hours, and responses can be obtained after a week, indicating very firm attachment at the site of injection. It was concluded that γ_1 antibody in the guinea-pig could be regarded as the equivalent of reaginic antibody in man; since both fix in skin and can provoke tissue reactions.

When large amounts of antibody are used, fixation by tissues may not always be necessary. Germuth and McKinnon (1957) showed that soluble antigen-antibody complexes formed in excess antigen and injected intravenously produced fatal systemic anaphylaxis in about one quarter of normal guinea-pigs, while insoluble

complexes at equivalence or in antibody excess, were inactive. Ishizaka, Ishizaka and Campbell (1959) produced permeability reactions without delay in normal guinea-pigs by intradermal injection of soluble antigen-antibody complexes.

They suggested that the reaction occurs as a result of molecular changes in the antigen or antibody molecule following their combination, which make the complex toxic. Broder and Schild (1965), using soluble antigen-antibody complexes prepared in excess antigen, demonstrated that when washed isolated normal guinea-pig lung was perfused with these complexes, broncho-constriction, and the release of histamine and SRS occurred. However these effects were antagonised by the presence of normal serum, which indicated competition or alternative sites for attachment, and suggested that the antigen-antibody complexes require attachment to the cells before producing the effects. An alternative possibility might be that serum contains enzyme in-activating factors. Insoluble antigen-antibody complexes were found to be 125 times less active than soluble complexes. This would be expected if it was assumed that antigen-antibody molecules

act by attachment to the cells and there activate enzyme systems (including complement), since large aggregates of antigen-antibody complex would present a much smaller number of active sites than dispersed molecules of antibody, each fully combined with antigen. Such reactions are unlikely to occur in vivo except possibly in anaphylactic shock, since the amounts of antigen needed are extremely large.

The choice of antibody for sensitizing human tissues

Allergic subjects show characteristic wheal and erythema, within a few minutes of intradermal injection of the offending substance. Prausnitz and Kustner (1921), showed that this type of skin sensitivity was associated with an antibody present in the blood, since serum would passively sensitize the skin of the normal recipients.

In 1962, Heremans and Vaerman showed that the P-K activity from the sera of grass pollen sensitive patients was present in fractions rich in γ_{1A}, and in 1964 Vaerman, Epstein, Fudenberg and Ishizaka demonstrated that the reaginic activity was present in carefully purified γ_{1A} globulin from atopic sera. Fireman, Vannier and Goodman (1963) observed that sera from three ragweed sensitive

patients were rendered inactive for P-K reactions, when their γ_{1A} globulin content was removed by precipitation with specific anti-human γ_{1A} serum. It was thus evident that γ_{1A} globulin contained the sensitizing antibody, and it seemed likely that other types of antibody might interfere with sensitization or produce reactions which were not usually present in clinical allergy. The use of mixed (e.g. serum) antibody would therefore tend to complicate the reaction in vitro and make interpretation difficult. It therefore seemed appropriate to use human secretions which are rich in γ_{1A}, and practically free from γ_2 immune globulins. The choice of colostrum or saliva appeared to be rational, (Sherman et al 1940: Hanson 1961: Kraus and Sirisinha 1962) and although colostrum is a rich source, it had to be excluded because there would be a limited and uncertain supply and each sample would differ. It also seemed likely to give difficulty in purification but had the compensating advantage of comparatively little bacterial contamination.

In 1963, Tomasi and Zigelbaum reported the selective secretion of γ_{1A} globulins in saliva. These authors showed that in six cases studied, all showed the presence

of γ_{1A}, only two cases showed small amounts of γ_{1M} and none contained any detectable γ_G. The removal of γ_M is a simple matter, whereas the separation of all the γ_G from the γ_{1A} is difficult. Saliva was therefore the logical choice as a source of reaginic antibody.

AIM OF THE PRESENT WORK

The detailed study of the reactions involved in human atopic allergies has been retarded by the lack of reliable in vitro procedures employing cells. It is complicated by our inability to immuno-chemically identify the reaginic antibody, as well as by the complex nature of the natural antigens. Various models of such systems using discrete cells, for example the agglutination of antigen coated red cells, (Gordon, Rose and Sehon 1958) or antigen induced histamine release from passively sensitized normal human leucocytes, (Van-Arsdel and Sells 1963), have proved not to be true models, or difficult to reproduce.

Information is now available concerning passive sensitization with reagins in vitro, employing animal materials, for example monkey ileum has been sensitized with sera from pollen sensitive individuals (Girard, Rose, Yagi and Abresman 1965). Human ileum, uterus (Tollackson and Frick 1966) and appendix (Chopra, Kovacs and co-workers 1965), have also been passively sensitized in vitro, but these tissues are obviously not suitable

for systematic studies, since a sufficient number of replicates cannot be obtained. There is broad similarity between severe allergic reactions in man and anaphylactic reactions in the monkey and guinea-pig, and isolated guinea-pig lung, particularly as small fragments maintained in physiological nutrient solutions, has been used extensively to study the dynamics of sensitization and the biochemistry of anaphylactic histamine release. As asthma is the most important single allergic condition in man, and the work in the guinea-pig would seem to be related to it, the human tissue having the best claim to further research would seem to be lung. Lung tissues from adequately documented asthmatic patients are very seldom available and will inevitably differ from each other. Passive sensitization in vitro could avoid many of these disadvantages, and experience with chopped guinea-pig lung could serve as a guide.

Once the passive sensitization of reasonably large samples of human lung had been achieved, it would be possible to compare the dynamics of the sensitization process in the human lung with the established facts

concerning guinea-pig lung tissue using similar methods. Work in the guinea-pig and the rat has shown that the different sub-classes of immune γ-globulins may each have an individual role in hypersensitivity reactions, and that work with single types of immune globulins becomes desirable. When whole serum is used there will always be uncertainty concerning possible interference between the constituent γ-globulins, both in diminishing sensitization, and also by complicating the biochemical processes triggered by antigen. The use of a single type of antibody, (e.g. reagin), and of a single defined antigen (e.g. a hapten) was therefore desirable.

It was intended to study:-

(1) Satisfactory conditions for the passive sensitization of human lung.

(2) The nature of the sensitizing antibody and the effects of other antibodies or serum proteins on sensitization.

(3) The inhibition of antigen-induced release of histamine as an indication of the biochemical steps underlying the reaction.

As a first approach, the penicillin system was favoured and saliva was to be the source of antibody.

SECTION I

Attempts to obtain γ_{1A} globulin and to sensitize human lung in vitro.

The source of the antibody had to be plentiful and as uniform as possible. For the reasons already mentioned saliva from persons sensitive to penicillin was chosen. It was expected that cooperative hypersensitive subjects would be easy to find and that all the antibody should react with the same antigen. It was however necessary to carry out trials for the purification of normal saliva before trying to isolate immune globulins from the atopic saliva.

Preparation of antibody

Collection and preliminary treatment of saliva

Saliva was collected from beneath the tongue by gentle suction, and passed directly through polythene tubes to an ice chilled glass container. It was centrifuged at 4°C for 30 minutes at 2000g. Salivary mucin was removed in the form of a clot by freezing and thawing the saliva, as described by Simons et al (1964). This product was called "whole saliva". Large volumes of

saliva treated in this way were concentrated at about 4°C by negative pressure dialysis, (Hofsten and Falkbring 1960), while smaller volumes were concentrated in the cold by Biodryex, using 18/32" Visking tubing.

Separation of Immune globulins

This was carried out by DEAE Cellulose chromatography. 1ml concentrated saliva (30-35mg protein content) dialysed against 0.01 M sodium phosphate buffer pH8 was applied to the column 1cm. diam. x 30cm. Peak I was eluted using the original 0.01 M buffer, and then a continuous gradient of increasing ionic strength (with NaCl) was established, using a mixing device of the sort described by Peterson and Sober (1959). The optical density of the eluates was measured at a wave length 280 mμ, using a Beckman DB Spectrophotometer. (Fig. 1).

The eluted fractions were identified by micro-immuno-electrophoresis, double diffusion technique, (p. 95, section 2, for detailed methods), and by starch gel electrophoresis as described by Bodman, (1960).

Whole saliva characteristically showed precipitation lines in the region of albumin and globulins, when a commercial goat anti-human serum (Hyland) was employed,

Fig. 1 .- DEAE Cellulose Chromatography.

Elution pattern of 1 ml concentrated saliva onto column 1 cm diameter x 30 cm. After peak I was obtained using sodium phosphate buffer, pH 8, 0.01 M, a linear gradient of increasing molarity was established. Protein concentration was taken as the optical density (1 cm) at 280 mμ.

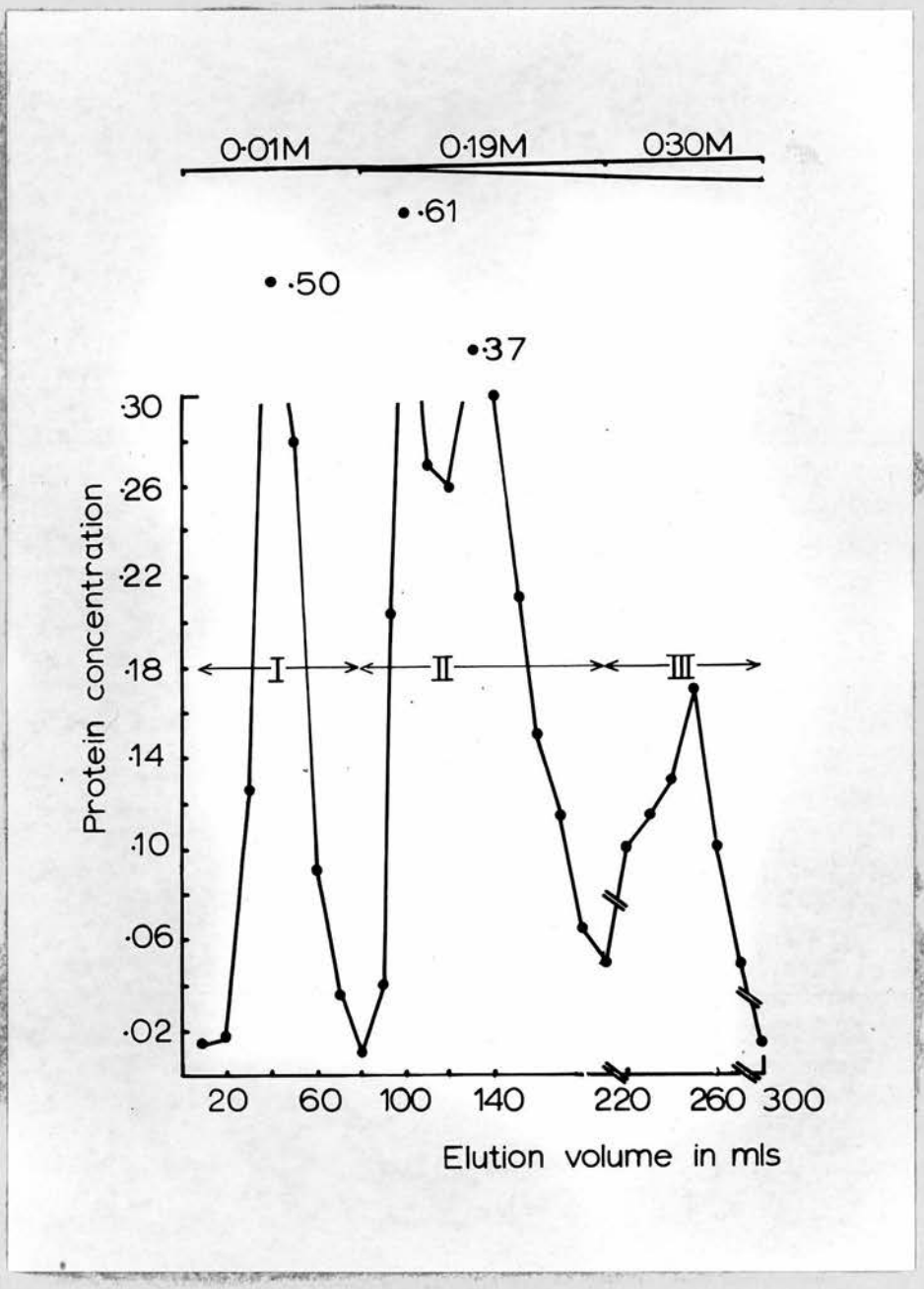

0·01M
0·19M
0·30M
·61
·50
·37
·30
·26
·22
·18
·14
·10
·06
·02
Protein concentration
I
II
III
20
60
100
140
220
260
300
Elution volume in mls

Fig: 2. Immuno electrophoresis of human serum and whole saliva:-

Top well = human serum

Trough = Goat anti whole human serum (HYL.)

Bottom well = whole saliva

Fig: 3. MIE of salivary peaks from DEAE-Cellulose chromatography.

Top well = peak II

Trough = Goat anti-human γ_{1A} serum (HYL.)

Bottom well = peak I

Fig: 4 Starch gel electrophoresis

Slit II = represents peak II

Middle Slit = shows bands of human serum proteins stained with 1% Bromophenol blue.

Slit I = represents peak I

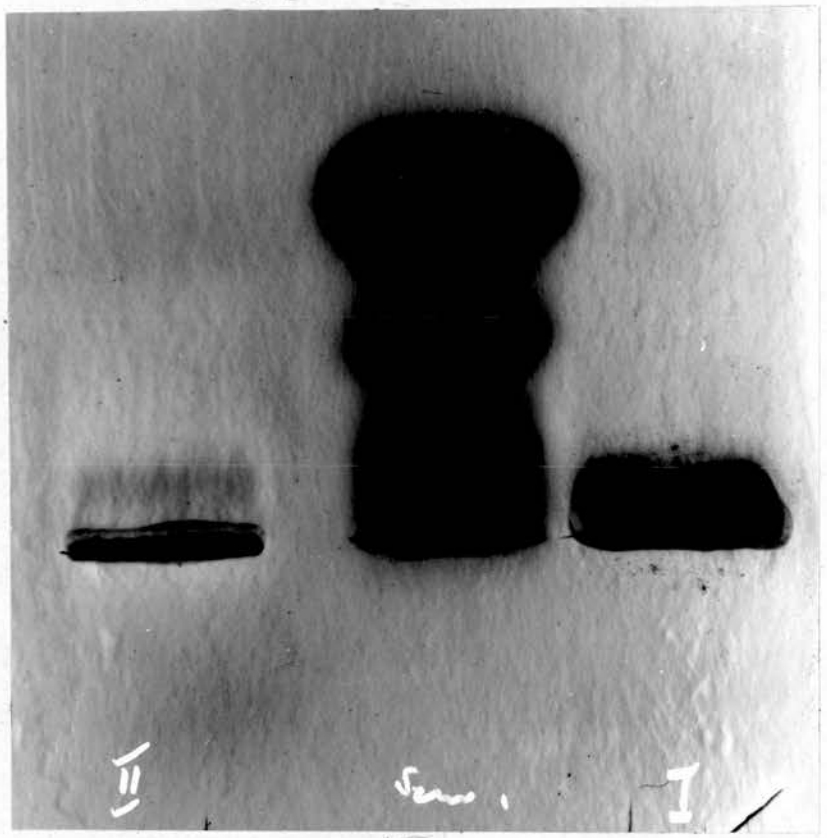

II
I

Fig: 5. Immunoelectrophoresis

Top well = γ_{1A} (serum)

Trough = Anti whole human serum (HYL.)

Bottom well = peak II (saliva) from DEAE col.

Fig: 6. Gel double diffusion

Central well = Goat anti whole human serum (Hyland)

Top well = peak I saliva

Left well = whole saliva

Bottom well = peak II saliva

Right well = γ_{1A} (serum)

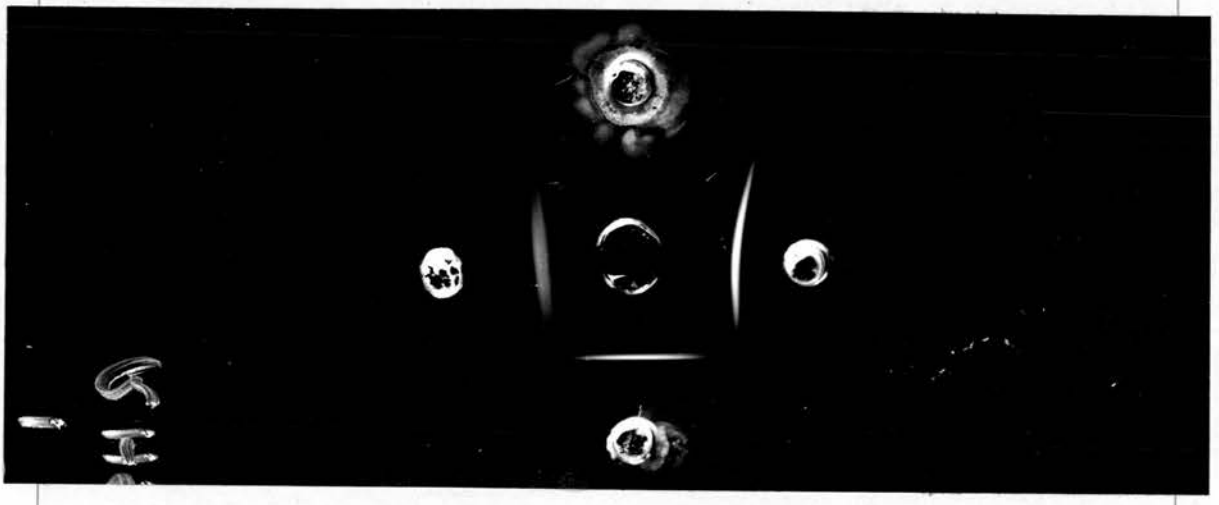


figure 2. Figure 3, demonstrates the presence of γ_1 in peak II, when goat anti-human γ_{1A} serum (Hyland) was used, while no line could be detected for peak I. However, electrophoresis of peak I on starch gel, (figure 4) shows digestion of starch, whereas peak II suggests the presence of slow β-globulin. Figures 5 and 6 confirm the identity of γ_{1A} globulin in peak II. It was concluded that peak II would contain the reaginic antibodies of the saliva and should be tested for its ability to give passive sensitization.

Preparation of antigen

Antigen protein conjugate was prepared by the method described by de Weck and Blum (1965). 250mg penicillin G, in 2ml distilled water was reacted with 120mg of human serum albumin in 4ml 0.1 M sodium phosphate buffer pH8, to which 1mg/ml EDTA was added. The mixture was incubated at 37°C, for 90 minutes. The unconjugated hapten was separated from the conjugate by gel filtration through Sephadex G 50 on a column 1cm. diam. x 30cm., the eluting buffer being sodium phosphate 0.01 M at pH8.

Passive sensitization of chopped lung

Dilutions of whole saliva and purified preparations of saliva from penicillin sensitive patients were employed for the passive sensitization of lung, and freshly prepared protein-penicillin conjugate was used to challenge the tissue. The experiment was conducted on lines identical to those found satisfactory in experiments using guinea-pig lung. (Brocklehurst and Colquhoun 1965). No histamine release was obtained. Failure to show sensitization was attributed to the materials rather than the method of sensitization. It was thought that the antibody might have been modified, or lost, or that the antigen was not satisfactory. Serious disadvantages were encountered in working with atopic saliva: viz.

(1) Very few adequately documented cases of penicillin sensitivity were available locally,

(2) collection procedure was very slow, and tiring for the patient,

(3) transport of saliva from distant hospitals may permit undue bacterial action before purification,

(4) it was difficult to ascertain if the penicillin

antibodies were associated with the salivary γ_{1A}, since these have not been well characterised.

In view of these uncertainties and problems, preliminary experiments were performed using serum from patients with a history of immediate systemic reactions to penicillin. These were intended to demonstrate the suitability of the antigen to combine with the reaginic antibody known to be present in the sera. When no significant sensitization could be detected, it was clear that although the antigen was the most likely source of trouble, there was no way to determine what was at fault. It was therefore decided to try some other antigen antibody system, in order to show that our handling of the tissue was satisfactory. The use of reaginic serum from pollen sensitive patients was determined by the offer of a collection of such sera by clinical colleagues, and a supply of antigen with which skin tests had been performed.

Satisfactory experiments were already being done by a group headed by Dr. R. C. Altounyan and Mr. P. Sheard of Fisons Research Laboratories, Holmes Chapel, Cheshire, by soaking tissue overnight, at room temperature. Some

of their strongly sensitizing serum and known antigen was given to us, but passive sensitization was not achieved using conditions which were successful in the guinea-pig work. The firmly held idea that passive sensitization of human tissue could be modelled on the guinea-pig lung sensitization therefore had to be abondoned. Suitable conditions for satisfactory in vitro sensitization of human lung were then investigated, (Section 2). Before doing this, one experiment was performed in an attempt to show that salivary antibody could be used for sensitization in vitro. The result is shown in Table 1. It was feared that whole saliva would encourage excessive bacterial growth during 18 hours at room temperature, so the tissue was soaked for 16 hours at 4°C and 2 hours at 37°C in the belief that this would prove to be superior. The routine procedure for challenge was employed. Only small amounts of histamine were released, and at the time the experiment was regarded as a failure, the tissue was blamed and the results ignored. It was realized later that the conditions of sensitization were at fault, and that the small amount of sensitization produced by the

serum was in line with other experiments run at 4°C. Sensitization by both serum and saliva reached the level of statistical significance, and the experiment thus indicates that sensitization with saliva is practicable.

TABLE 1

The use of whole saliva and whole serum from two patients sensitive to cocks-foot pollen to sensitize human lung in vitro

Uptake of antibody 16 hours at 4°C + 2 hours at 37°C . Challenge at 37°C in Tyrode solution + aqueous extract. ("Pollacine") = pollen 1 in 10,000, for 15 mins. Total tissue histamine at end of period = 24.1µg/g. The histamine released is stated as % of that present: the standard deviation and number of replicates are shown.

Treatment during uptake	Challenge	Histamine released	Statistical Comparison	
1) Tyr.	Tyr.	4.5 ± 0.59 (4)	1V2	.3>P>.2
2) Tyr.	Ag.	3.5 ± 0.45 (3)		
3) Ab. (ser.)	Tyr.	3.7 ± 0.48 (5)	1V3	.4>P>.3
4) Sal. (1:20)	Ag.	5.4 ± 0.2 (7)	2V4	.01>P>.005
5) Ser. (1:40)	Ag.	6.1 ± 0.36 (9)	2V5	.005>P>.001

SECTION 2

Development of a reliable procedure for passive sensitization of human lung.

Introduction

After it had been confirmed that chopped human lung could be sensitized with reagin-containing antibody by prolonged soaking, it was necessary to produce a stock of suitable serum so that a series of related results could be obtained. It was decided first to test a large number of serum samples to find which were effective when applied for 18 hours at laboratory temperature. These conditions for sensitization had been arbitrarily used for convenience, and were not varied during the preliminary experiments. Later it was necessary to establish the best conditions of time and temperature during the period of sensitization and up to the time of challenge, and it was found that these conditions were very satisfactory.

In early experiments, the tissue was cut into rods 0.4 x 0.4 mm as is commonly done with guinea-pig tissue. The human tissue proved to be rather slimy and tended to fragment so that it was difficult to filter off the supernatant fluid, and the size was increased to 0.8 x 0.8 mm to make it easier to handle. It was argued that

the uptake of antibody would not be influenced because of the long period of soaking, and that the penetration of antigen could be checked by the time-course of histamine release at a later date. The concentration of antigen used initially was "Pollaccine" 1 in 200. (i.e. = 1 mg pollen in 2 mls or 1 in 2000). It was found that 1 in 2000 (i.e. = pollen 1 in 20,000) was equally effective as an antigen, and for routine use 1 in 500 "Pollaccine" (= pollen 1 in 5,000) was used to give a margin of safety when unknown antisera were being tested or higher concentrations of the 'standard' antiserum were used.

The period of incubation with antigen and of pre-warming to 37°C was based on experience with guinea-pig tissue. To avoid error should these conditions not be ideal, the times and temperatures were rigidly adhered to. The time for release was purposely kept rather short to minimize the amount of histamine released spontaneously in the control samples.

The relevant details of the procedure used in early studies was as follows:-

(Note: the method adopted finally is described on p 56)

The tissue was collected in oxygenated Tyrode solution, the pleura was removed and tissue chopped into rods of 0.8 x 0.8 mm., The tissue was soaked in reaginic serum containing antibodies against cocksfoot. The serum most frequently used was Stopford Serum (SS_1) in a dilution of $^1/_{40}$. After soaking at 17°C for 18 hours the tissue was washed, incubated for 5 minutes and challenged with antigen, Pollaccine (1 = 500) at 37°C, with continuous rocking for 15 minutes. The histamine content of the tissue and the supernatant was determined. Parallel controls were run to ensure that the release was due to an antigen antibody reaction on the tissue.

The methods used for the bioassay of released substances are well-known; details are given on pages 57 to 58.

Serum Samples

(a) Reaginic Sera

Blood samples were obtained in sterile containers from patients who were found to be strongly positive by the direct wheal-and-flare reaction to an aqueous extract of the pollen. None of the cases had ever received previous specific hyposensitization therapy. The blood

was allowed to clot, and the serum obtained by centrifugation at 4°C for 15 mins. at 1600g. The serum samples were stored at -20°C until used.

(b) Normal human serum

Blood samples were obtained from healthy adult volunteers who had no previous history of hay-fever or asthma, and the serum was obtained in the manner as for the reaginic sera.

HUMAN LUNG SAMPLES

Source of material

Samples of human lung were obtained from the thoracic surgery units at the Royal Infirmary and City Hospitals in Edinburgh. The tissue used usually showed no evidence of gross deterioration or malignancy, and was usually from the peripheral part of a lobe which had been excised because of a growth involving the bronchus. The specimen was placed in chilled Tyrode solution (about 10°C) very shortly after excision and used within 2 hours.

The external appearance of the specimen varied from pink, spongy, moderately anthracotic tissue, with thin, smooth pleura, to mottled, greyish black and puckered tissue, with thick, tough pleura, and the remains of

fibrous adhesions. The pathologists' reports of the specimens used in this study show that about 80% of the cases were grouped as bronchogenic carcinoma of squamous cell type with a few cases of oat cell carcinoma. A single specimen was obtained from a case, in which lobectomy was performed for malignancy, but was later diagnosed as a case of organizing pneumonia. No experimental differences were noticed with samples from the various cases, provided that the tissue was sufficiently healthy to retain histamine.

As a routine the patients did not receive any preoperative medication. The anaesthesia administered consisted of a mixture of nitrous oxide, oxygen and fluothane. The patients were predominantly males, aged 40 to 68 years, usually over 50 years old. The patients came from all walks of life including school teachers, bakers, farmers and textile mill workers. All the patients were in reasonable health, apart from complaints pertaining to the respiratory system. Sensitivity to penicillin was alleged in a few of the patients but no other hypersensitivity was noted.

In one case special enquiries were made to ascertain

if the patient was sensitive to fungi; (refer to discussion on p. 88)

The selection of suitable tissue from the lung

In a lung the size and age of those usually available; the pleura is extremely tough by comparison with the guinea-pig, and there will be rather large bronchioles with tough cartilage in all parts other than the periphery. Only the lung parenchyma afforded convenience in cutting, and the possibility of uniform samples of tissue, freely accessible to both antigen and anitbody. The suitability of this tissue for routine use was shown by an experiment in which tissue containing lung parenchyma, containing a few small bronchioles, was compared with peripheral parenchyma with attached pleura, for ability to release histamine after the usual procedure of sensitization and challenge.

The results shown in Table 2 , make it clear that the lung parenchyma is well suited to the purpose. The amount of histamine present in the parenchyma was surprisingly high, exceeding that in the samples containing pleura where most of the mast cells are commonly supposed to be found. The tissue did not retain histamine aswell as guinea-pig lung, but this might be the result of

TABLE 2

The Effect of the presence of pleura in the samples of chopped lung used in passive sensitization

Sample A contained only lung parenchyma from peripheral sites. Histamine 10.9µg/g.

Sample B contained pleura with the adjacent lung parenchyma attached. Histamine 7.4µg/g.

The samples were from adjacent parts of the same lobe, and were treated identically.

		% Histamine release
A	control	10.2, 9.9, 9.4
	sensitized	14.5, 15.6, 9.2
B	control	14.6, 12.5, 11.2
	sensitized	8.2, 12.6, 12.2

using material removed at operation and not perfused or chopped immediately. Surprisingly, the spontaneous release of histamine was greatest from the sample containing pleura. In routine experiments the pleura was dissected off the lung, and slices of the underlying tissue cut about 5 mm thick and then sliced into rods 0.8 x 0.8 mm.

The storage of chopped lung

Lung cannot be obtained very frequently or with certainty and it would be an advantage if tissue could be stored. It also seemed possible that soaking prior to sensitization might improve the condition of the tissue by removing traces of fluothane or other substances which it might contain as a result of the operation. Tissue was therefore chopped and stored at 4°C. for 24 hours and then compared with the same tissue used fresh.

The results are given in Table3 , which also shows the effect of using a very potent reaginic serum and a weaker one to produce sensitization. The histamine content of the tissue fell by about 10% on storage, but this may have come from damaged cells since the spontaneous release from the soaked tissue was low. Evidently tissue can be stored in this way, but there is no advantage which would justify storage as a routine procedure.

TABLE 3

The effect of storing chopped lung tissue in an excess of Tyrode solution without added oxygen at 4°C for 24 hours on subsequent sensitization by two reaginic sera.

Tissue	Treatment with antigen and antibody	µg Hist. / g Tissue	% Hist. released
FRESH	Tyr. + Tyr	15.75	3.1
	Tyr. + Ag.		2.9
	$Ab_{(A)}$ + Ag.		11.3
	$Ab_{(B)}$ + Ag.		20.7
STORED	Tyr. + Tyr.	14.28	1.8
	$Ab_{(A)}$ + Ag.		6.2
	$Ab_{(B)}$ + Ag.		19.4

The variability of results due to the use of tissue which is not healthy.

The supply of lung was not sufficiently plentiful to allow the rejection of all tissues having an abnormal appearance. Table 4, shows the results of treating different tissue samples by the routine procedure for sensitization and challenge. The tissues include those which would be rated "good" and "poor" on the basis of appearance. Experience has shown that a smooth, thin pleura is probably the best superficial indication that the tissue is satisfactory, but the table shows that some apparently unwholesome samples have proved to have a good content of histamine which was well retained, and have permitted useful experiments.

Example 9 in the table shows that some experiments failed because the tissue was bad. Example 8 illustrates the possibility that some samples came from subjects who were subclinically sensitive to the antigen used.

TABLE 4

The gross appearance of lung samples used, and the anaphylactic release of histamine obtained in a routine procedure of sensitization and challenge.

Sensitizing solution, serum 2, 1 in 20.
Antigens= cocksfoot, defatted total aqueous extract
= pollen 1 in 10,000.

TABLE 4

The variability in gross appearance of the samples of lung tissue, and the anaphylactic release of histamine obtained with the same sensitizing antibody and the same antigen.

Tissue samples:		% Histamine released from tissue		
Gross appearance	Tissue hist. ug/g	Spontaneous	Antigen induced spontaneous	Anaphylactic histamine release
1 - Sooty; inflamed pleura; thickened bronchioles.	9.7	6.5	7.5	5.4
2 - Spongy tissue; thick pleura	10.2	4.9	4.3	12.0
3 - Pinkish, spongy tissue; thin smooth pleura	15	3	2.9	17.1
4 - Pink spongy tissue; thin smooth pleura	80	1.8	2.1	38.8
5 - Sooty patches; spongy; smooth pleura	14.1	-	4.9	46.5
6 - Sooty patches; spongy; smooth pleura	11.5	8.8	9.1	16.4
7 - Sooty patches; spongy; thick soft opaque pleura	21.7	-	4.9	5.8
8 - Sooty; very slimy; soft jelly-like thick pleura	7.1	1.7	5.2	6.1
9 - Tissue brown coloured; thick tough pleura	6.3	-	7	(1)

EXPERIMENTS TO DETERMINE SUITABLE CONDITIONS FOR STUDIES CONCERNING IN VITRO SENSITIZATION OF CHOPPED HUMAN LUNG.

Effect of temperature and time of uptake on passive sensitization.

Table 5 shows the effect of varying the conditions of antibody uptake on the spontaneous and anaphylactic histamine release from chopped human lung. It is evident that sensitization as indicated by the anaphylactic histamine release is satisfactorily achieved in tissue maintained at 17°C for about 18 hours. At a temperature of 4°C detectable sensitization occurs if the tissue is soaked in serum for about 24-36 hours. Sensitization comparable to that produced in 18 hours at 17°C requires about 72 hours soaking at 4°C. The spontaneous histamine release in the cold is always high, and the remaining tissue histamine is correspondingly reduced. By contrast, at 37°C the tissue histamine is well retained, and the controls are reasonable, but adequate sensitization is not obtained in 4 hours.

Fig (7) represents the time course for the in vitro passive sensitization, spontaneous release, and total tissue histamine of two lung samples. It shows that a

TABLE 5

The sensitization of chopped human lung by soaking in atopic serum. The effect of temperature and time on the condition of the tissue and the level of sensitization reached.

Results from 4 experiments using different samples of tissue and sensitizing serum. All are mean values of triplicate tests. Anaphylactic release of histamine during 15 minutes at 37°C antigen - "Pollaccine" = pollen 1 in 10,000. Total histamine as µg/g wet tissue.

Experiment	Conditions for uptake		% Histamine release		Total tissue histamine at time of challenge
	Temp.	Hours	sensitized	control	
1	4°C	36	18.9	13.7	17.4
2	4°C	72	21.1	13.5	6.7
		90	26.0	13.6	6.4
		120	30.3	11.8	4.8
1	17°C	18	22.4	5.7	15.5
2	17°C	18	20.4	10.6	9.4
3	17°C	3	6.1	5.4	22.3
		18	21.5	5.6	16.4
4	17°C	18	22.8	9.7	10.9
3	37°C	2	5.5	5.2	19.5
4	37°C	4	10.1	6.7	12.7

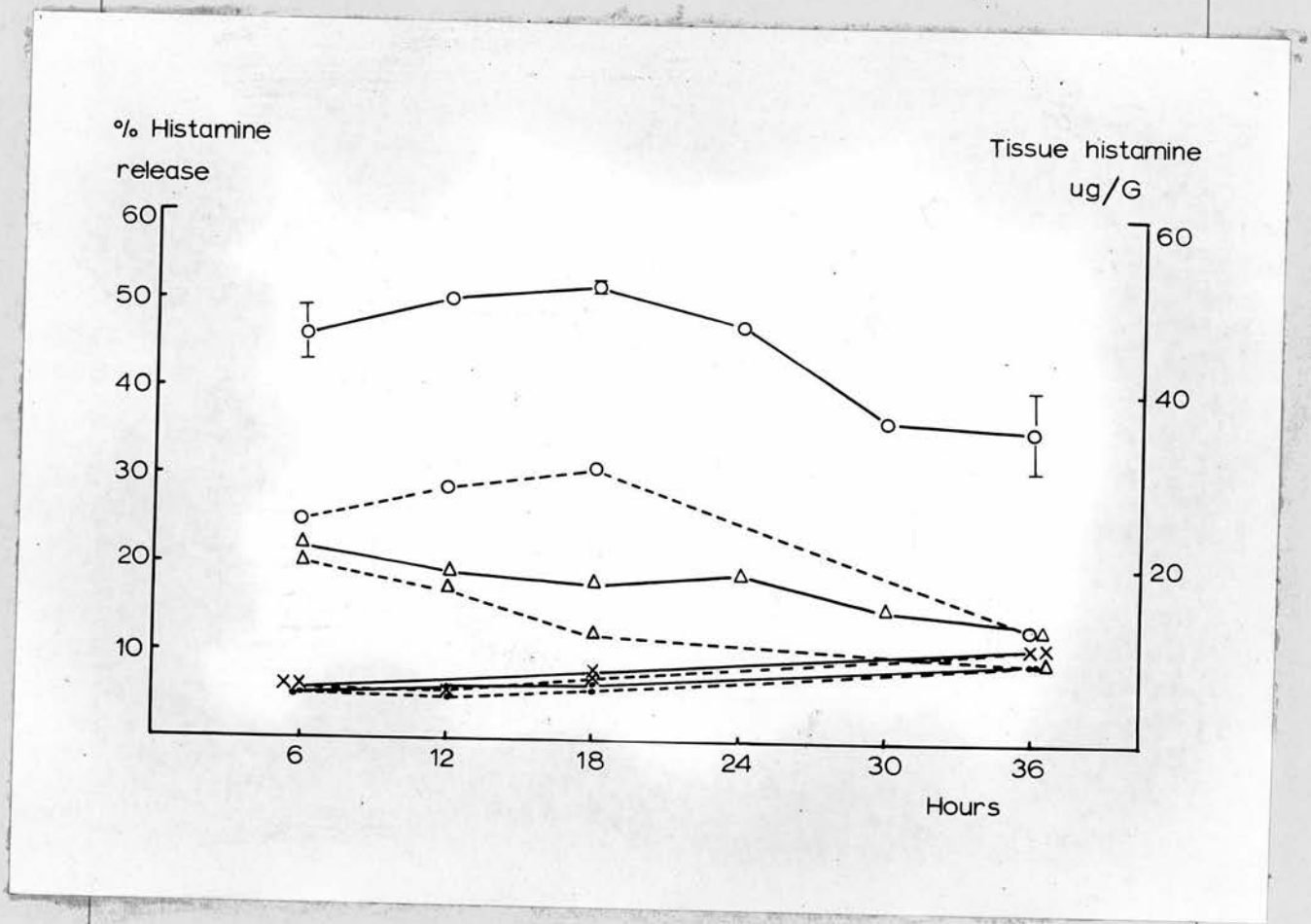
Fig. 7 .- Determination of the optimal time for passive sensitization of chopped human lung at 17°C.

Two specimens of lung used, o—o and o---o. Sensitization assessed as the % histamine released on challenge by the standard procedure (p. 57). Spontaneous release of histamine (•·····•).

Histamine released by antigen, from non-sensitized tissue ×····×

Total histamine µg/g tissue at end of sensitization period Δ·····Δ

S.E. bars calculated from triplicate samples.


% Histamine
release
Tissue histamine
ug/G
60
50
40
30
20
10
60
40
20
6
12
18
24
30
36
Hours

period of 12-18 hours is necessary to reach a steady high level of sensitization, and that at this time, tissue kept at 17°C remains in a satisfactory condition although histamine is being lost continually. It would seem that 12 hours soaking is appropriate, and that appreciable sensitization has been produced in 6 hours, with the very good sample of antibody serum used, after 18 hours a further increase of sensitization is not found, and deterioration of the tissue becomes serious.

The effect of incubation at 37°C after uptake at 17°C

Fig (8) illustrates that sensitization is not improved when the tissue is incubated in the antiserum at 37°C, after an initial period of 18 hours soaking at 17°C. This result was not anticipated; indeed it had been hoped that sensitization would increase. Part of the reduced anaphylactic release of histamine from the tissue will be due to the high loss during incubation. This is at the rate of about 16% per hour, so that after 120 minutes at 37°C approximately 30% of the histamine is lost. The loss at the end of 18 hours soaking at 17°C is only about 1.5% per hour. The loss of histamine from the tissue during incubation at 37°C may not fully

Fig. 8 .- The effect of subsequent incubation at 37°C upon the sensitization produced by soaking the tissue in antiserum at 17°C for 18 hours.

Two tissues (a) denoted x⟷x , 80 µg histamine/g and (b) denoted x·····x, 13 µg histamine/g.

Two antisera (a) in a dilution of 1 in 80, and (b) 1 in 40.

Anaphylactic release of histamine during 15 mins following challenge with antigen (Pollaccine = cocksfoot pollen (1 in 10,000) denoted X - X, spontaneous release O- O.

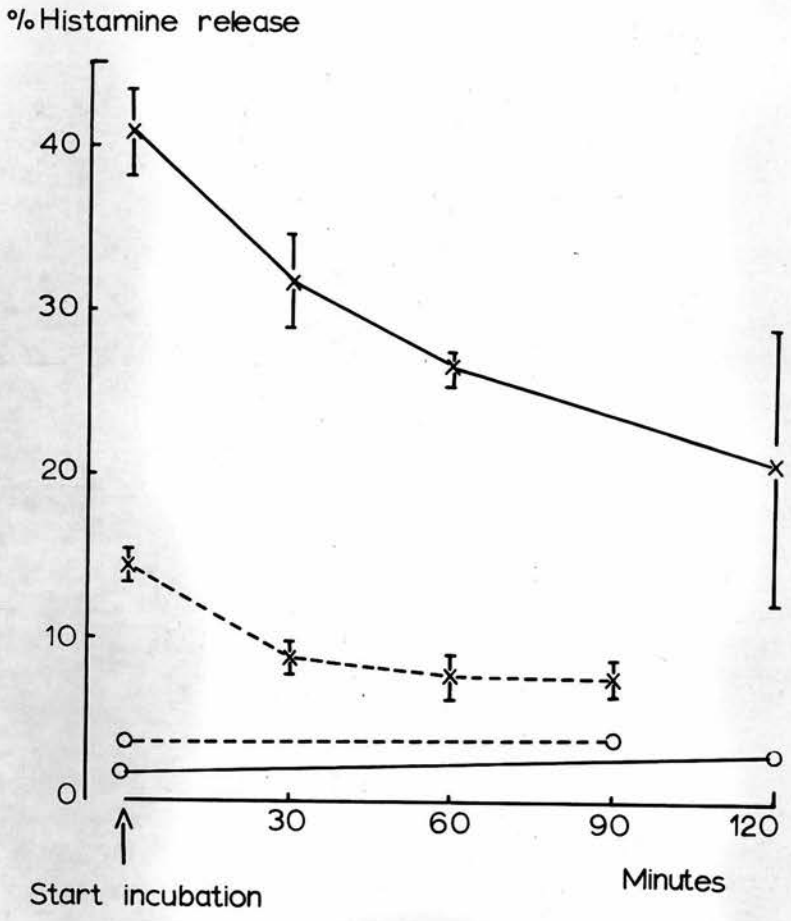

% Histamine release
40
30
20
10
0
30
60
90
120
Minutes
Start incubation

TABLE 6

Effect of changing the temperature for part of the sensitization period

antibody solution = serum 40, 1 in 20.

antigen = cocksfoot aqueous extract = pollen 1 in 20,000

standard uptake period = 18 hours at 17°C.

S = Tyrode solution + non-sensitized tissue

C = antigen + non-sensitized tissue

A = antigen + sensitized tissue.

(All during 15 minutes "challenge" period at 37°C).

Treatment of Tissue	Challenge	% Histamine release	
I Incubated 30', 37°C before standard uptake	S	11.7, 14.5	
	C	11.7, 11.6	mean 11.6
	A	28.7, 31.3	" 30
II Incubated 15' 37°C with antibody after standard uptake	S	18.4, 15.2	mean 16.8
	C	18.4, 15.2	
	A	28.7, 42	" 35.3
III Standard uptake	S	10.2, 9.5	" 9.9
	C	14.3, 15.3	" 14.9
	A	48.5, 53.6	" 51

account for the 50% reduction in release on challenge.

Table 6 supplements fig. 8 and mainly concerns the effect of a short period of incubation at 37°C with antibody after the usual long soaking period at 17°C, (procedure II). This experiment shows that such a procedure is disadvantageous. The histamine content of the tissue was reduced from 18µg/g to 16µg/g by incubation at 37°C before or after soaking at 17°C (I and II), compared with that of the tissue treated by the standard procedure (III). This loss is too small to account for the reduction in histamine release on challenge, when expressed as absolute quantities, and the change must reflect a less effective anaphylactic reaction.

The amount of histamine released during anaphylaxis in vitro was shown by Mongar and Schild (1957) to be affected by temperature, and to require metabolically competent cells. Since the sensitization in vitro of chopped human lung involves long periods at low temperature (17°C), and possibly to low oxygen tension, it was necessary to establish whether a period of incubation to give a steady temperature of 37°C and to restore the metabolic activity of the tissue prior to challenge was important for anaphylactic activity.

TABLE 7

Effect of varying "pre-challenge" and "challenge" temperatures upon histamine release from chopped human lung tissue passively sensitized at 17°C.

Total histamine 16.4 µg/g tissue.

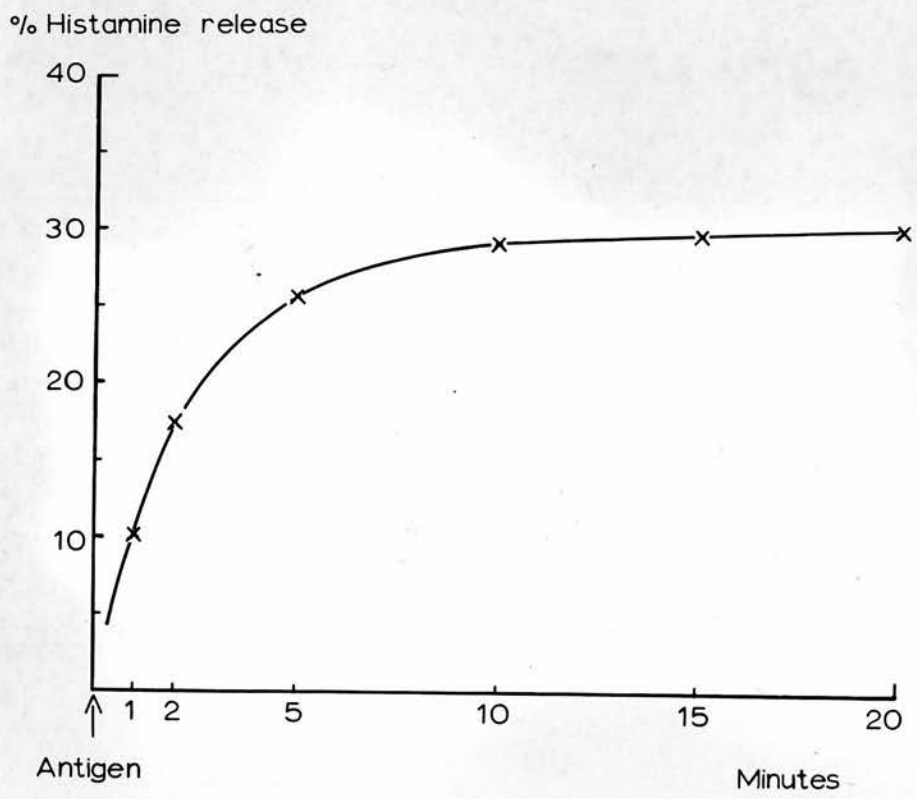
5 mins prior to challenge	Challenge		Histamine release (Percent)
	Temp. C.	Time mins	
17°C.	17°	15	8.2
17°C.	37°	5	15.8
		15	17.6
37°C.	37°	1	10.5
		5	19.4
		15	23.6
Control	37°	15	5.6

Table (7) shows that the greatest release of histamine occurs when the antigen is added at 37°C to tissue already held at this temperature for several minutes. The release is rapid, being easily detectable within the first minute, and 75% complete in 5 minutes. If the temperature of the tissue is 17°C before challenge, and at challenge rises to 27°C and then fairly rapidly to 37°C, there is still a substantial histamine release, (i.e. 70% of the release at 37°C) at the end of 15 minutes. This rather high value suggests that the tissue has not lost its ability to react as a result of incubation at 17°C, and confirms that challenge at 27°C is sub-optimal. The effect of incubation at 17°C at all times was to practically abolish the anaphylactic reaction as expected. It was concluded that pre-incubation at 37°C for 5 minutes was adequate, and this is supported by the results in Table 6 , which indicate that 20 minutes incubation (II) has increased the rate of spontaneous histamine release but diminished the anaphylactic release.

<u>The time-course of histamine release following challenge at 37°C</u>

According to Austen and Brocklehurst 1961, two thirds of the total anaphylactic release of histamine took place

Fig. 9 .- The time course of histamine release after addition of antigen. Aliquots of supernatant fluid tested at intervals shown, the results integrated to give the curve.


% Histamine release
40
30
20
10
1
2
5
10
15
20
Antigen
Minutes

in the first minute of contact between the antigen and sensitized guinea-pig lung. Fig 9 shows that passively sensitized human lung, release 35% of the final amount of histamine in the first minute and 85% by the end of five minutes, and that only 3% is released between the 10th and 20th minute. Thus the time course for the anaphylactic release of histamine from the chopped human lung is practically identical to that observed with chopped guinea-pig lung, in spite of the use of slightly larger fragments of tissue. It was concluded that release was complete in 15 minutes, and this time of incubation with antigen was used routinely.

Relationship between sensitization and the concentration of applied antibody.

It is well established that sensitization increases with the concentration of the sensitizing antibody, and the time of contact. Fig. 10 (experiment △) shows a linear relationship over a limited range between the concentration of the antibody and amount of histamine released from the chopped human lung tissue on challenge.

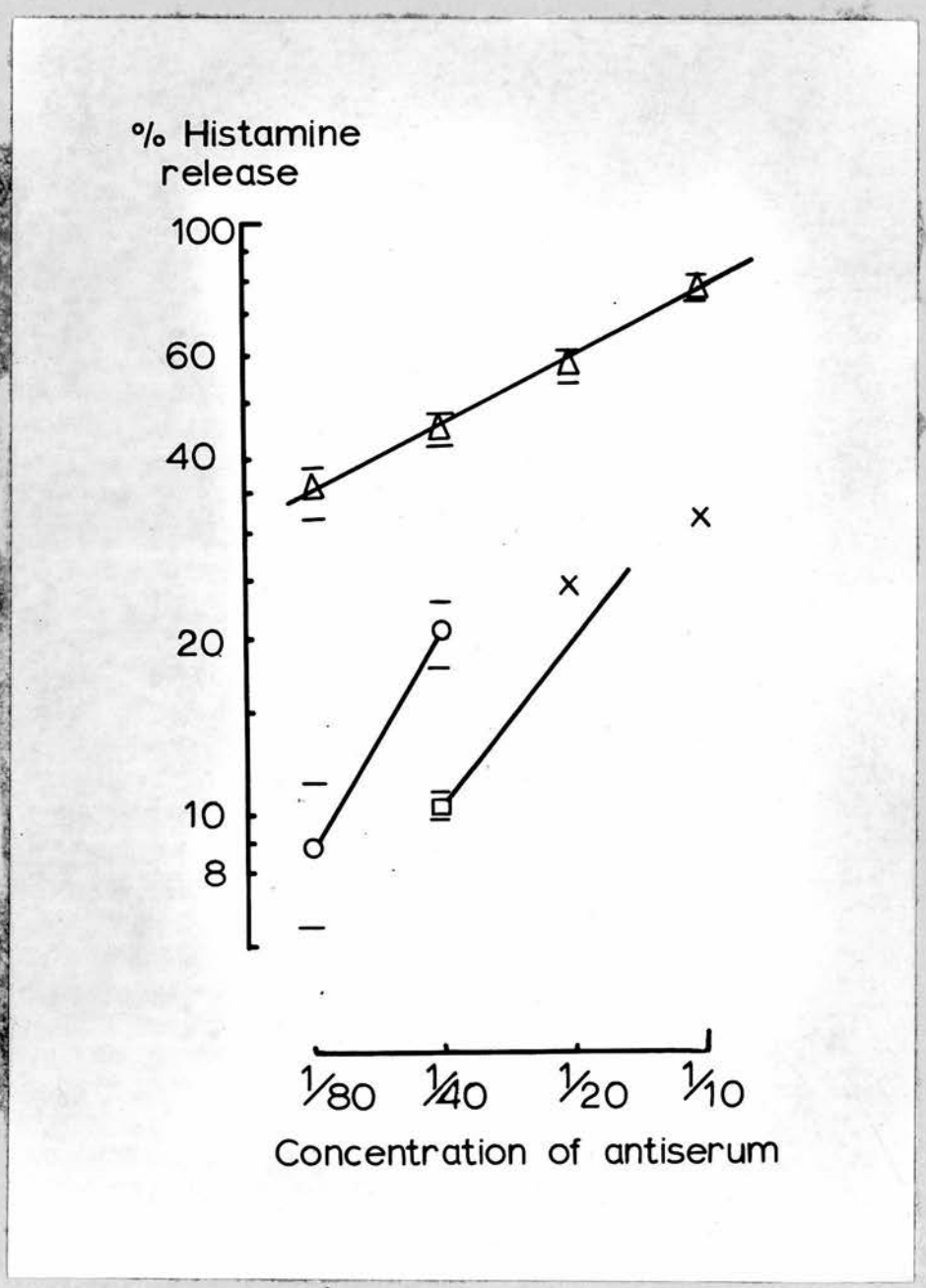
In this experiment 80% anaphylactic histamine release was obtained from the chopped lung using a potent atopic serum in $\frac{1}{10}$ dilution, and 30% histamine was released after using $\frac{1}{80}$ dilution. Other antisera could yield only about 20% histamine release with $\frac{1}{40}$ dilution.

The use of antisera active at high dilutions tends to avoid interference by other constituents of the serum and thus makes interpretation of results easier. The rather flat dose/effect slope and the small scatter of values with the potent antiserum provided a suitable basis for studies on inhibition of histamine release. Unfortunately sera of this potency are scarce.

The choice of solutions for soaking tissue.

The amount of histamine lost from the tissue during soaking at 17°C was regarded as acceptable when Tyrode

Fig. 10 .- Relationship between sensitization (antigen induced histamine release) and concentration of applied antibody. Three experiments using different lung tissues and atopic sera. Samples of lung tissue were exposed to dilutions of whole serum for 18 hrs at -17°C. The results are plotted on logarithmic scales with SD indicated for triplicate values. (Points marked X are the highest of two divergent values obtained.)


% Histamine release
100
60
40
20
10
8
1/80
1/40
1/20
1/10
Concentration of antiserum

solution was used, but the loss during re-warming and incubation at 37°C for 15 minutes (as during challenge) was usually in excess of 5%, and was considered to be too high. This loss must indicate that after 18 hours soaking in Tyrode solution the tissue is starting to deteriorate. Different physiological saline solutions were therefore tested, and Tyrode solution was modified by changes in Ca^{++}, and by the addition of a little EDTA (1:10,000) to remove toxic cations such as Cu^{++} and Pb^{++}. The addition of extra glucose was also tried. Later, in an attempt to reduce the presumed loss of protein from the surface of the cells, albumin and other fractions of human serum proteins were added to the fluid. The solutions were used during the whole period, i.e. soaking at 17°C for 18 hours, re-warming at 37°C for 5 minutes, and 15 minutes incubation at 37°C. The amount of histamine remaining in the tissue after 18 hours at 17°C was not greatly different in any of the solutions tested. The release during the 15 minutes corresponding to the challenge period is summarized in Tables (8) and (9). These show that no solution to the problem was found. It was concluded that the loss, which varied very widely, was probably the result of disease, or damage by trauma or

TABLE 8

The effect of various solutions on spontaneous release of histamine from chopped human lung. Three experiments.

Suspending Solution =	% Spontaneous his. release.
(1) Tyrode	8.8
Tyrode + 2 Ca^{++}	9.0
Kreb's (1950)	9.8
Kreb's Hens.	10.3
(2) Tyrode	11.3
Tyrode $\frac{1}{2}$ Ca^{++}	18.3
(3) Tyrode x 2 glucose	3.5
Kreb's (1950) x 5G sucrose	5.5
Kreb's Hens.	5.1
McEwen's solution	5.1
All these solutions contained 1=10,000 EDTA tetra-Na salt.	

TABLE 9

The effect of added protein during soaking at 17°C on the spontaneous release of histamine from chopped human lung.

Two Experiments

Treatment	ng histamine released/sample	% Histamine release	Comparison with Tyrode
(1) Tyrode solution	84	8.4	100
Tyrode+HSA 125 µg	92	9.2	109.5
250 µg	93	9.3	110.7
Tyrode + γ_G 25 µg	100	10.	119
50 µg	96	9.6	114.3
(2) Tyrode solution	68	4.7	100
Tyrode + γ_G 1mg	87	6.1	129.8
+ γ_A 1mg	86	6.1	129.8

chemicals, at or prior to excision, and that the loss must be accepted, since it did not seem to seriously alter the anaphylactic reaction. It seemed unwise to change from Tyrode solution, since this had been routinely used in all the comparable studies on guinea-pig tissue, is stable, and was known not to react with the substances to be tested as inhibitors later in the research.

SELECTION OF SUITABLE REAGINIC SERA

Preliminary screening of samples

A clinical colleague, Dr. I. W. B. Grant had collected a large number of samples of serum from patients in the Allergy Clinic, Northern General Hospital, Edinburgh, and provided small amounts of these from subjects sensitive to grass pollen. None of these had been actively hyposensitized, but many were treated with corticosteriods. All were taken from subjects giving strong positive skin tests to "Bencard" B_2 mixed grass pollen test solution (Cocksfoot, Timothy, Fescue and other grasses). The sera were stored at -20°C without preservative and were never thawed on more than two occassions before use. The test was performed by the standard procedure (page 56) using aqueous extract of defatted Cocksfoot pollen (equivalent to 1g pollen in 10,000 mls), described on page 52 .

In the main study, 46 sera were tested and gave the results summarized in Table (10). The classification of sensitivity is quite arbitrary, but only sera in the upper group were used in inhibition studies (section III). Each experiment included tests with a known potent serum used as an internal standard so that results obtained in

TABLE 10

Classification of reaginic sera by their ability to sensitize human lung at 1 in 20 dilution in a standard procedure

Histamine released	Number of samples
not dectectable (<4/5 control)	11
⩽10% of total content of tissue	18
10 - 30%	10
>30%	7

the series of experiments could be combined. By this use of a standard, unsatisfactory tissue was recognized and poor experiments were then repeated.

'Good' samples of serum were distributed into 1 ml lots in small tubes to avoid the need to thaw and re-freeze. These tubes were kept at -20°C.

VARIATION IN THE SENSITIZATION PRODUCED BY THE SAME ANTIBODY

It is inevitable that the antibody samples from patients will differ widely both in overall potency and probably in the range of antigenic constituents of the pollen with which they will react. Table 11 contains the collected results obtained with 6 different antisera all of which were rated "good" in the screening tests for potency. These results were obtained by the standard procedure for sensitization (page 56) in experiments to show the effect of modifying it, as reported in section II. The range of tissue histamine is enormous (5 to 80 μg/g) but a few values fall outside the range 12 to 30 μg/g, which corresponds with values common in the guinea-pig. The percentage of histamine released is not related to the

TABLE 11

Variations in the degree of sensitization which result from the biological characteristics of the lung tissue

Expt.	Antiserum No.	x dilution	Total Hist. µg/g	Anaphylactic Histamine %
9	SS 1	40	13.04	14.5
12		40	9.7	12.9
17		40	15.3	20.7
19		40	11.4	25.6
"		80	11.4	14.4
20		80	80.3	40.9
21		40	36.06	13.6
22		40	27	38.4
17	SS 100	40	15.3	11.3
21		40	36.06	10.4
23		40	14.1	51.4
24		40	11.5	19.6
"		20	11.5	25.5
"		10	11.5	32.2

Continued.

TABLE 11 (Continued)

Expt.	Antiserum No.	x dilution	Total Hist. µg/g	Anaphylactic Histamine %
42	SS 63	20	17.9	49.1
44		20	12.1	26.3
45		20	15.3	19.9
46		20	5.5	15.9
47		20	15.2	53.6
48		20	9.8	20.4
49		20	10.9	41.5
51		20	15.5	22.4
36	SS 40	20	27.1	29.9
37		20	7.47	32.1
38		20	10.9	23.7
39		20	11.3	44.7
40		20	15.6	51
41		20	12	31
42		20	17.9	51.9
43		20	6.5	50.2
44		20	12.1	23.8

Continued.

TABLE 11 (continued)

Expt.	Antiserum No.	x dilution	Total Hist. µg/g	Anaphylactic Histamine %
36	SS 82	20	27.1	14.5
45		20	15.3	16
50		20	16.4	21.5
51		20	15.4	26.3
42	SS 52	20	17.9	47.3
43		20	6.5	25.1
44		20	12.1	21.9
51		20	15.5	15.1
52		20	12.9	18.2

total histamine present, and must therefore be determined by the degree of sensitization produced. The only variable to account for these results is the tissue itself. As far as was known at the time of the experiment none of the tissues came from individuals who were allergic or who had abnormal patterns of γ globulins, but such details might well influence the degree of sensitization. The release of 41% histamine from very clean and apparently healthy tissue containing 80μg/g, following sensitization with 1 in 80 dilution of the antiserum clearly indicated a highly reactive tissue, and would be expected to produce some recognizable effect in the patient.

These results illustrate the need to have a reference serum whenever fresh samples of reaginic serum are being tested. It is also necessary to set some arbitrary level of response below which the experiment will be discounted as due to failure of the tissue. If this is not done, useful antisera may be discarded in error.

Samples from same subjects on two occasions.

Patients who had provided 'good' antisera in the screening tests were asked to give 20 mls of blood during attendance at the clinic. The intervals varied from 3

TABLE 12

All sera tested at dilution of 1 in 20. SS (40) is a laboratory reference sample. Mean values of triplicate tests.

Reaginic Serum	Anaphylactic histamine release	Details of donor
SS (40)	25.7% (of 27µg/g)	samples from patient DR sample (Y) was taken 4 months after (X): treatment, prednisolone.
DR (X)	10.3	
DR (Y)	10.1	
SS (40)	17.2% (of 15µg/g)	Samples from patient NO B taken 6 months after A, and following a clinically successful course of hyposensitization
NO (A)	14.5	
SS (40)	25.7% (of 27µg/g)	
NO (B)	1.6	

months to one year. All new samples of serum were tested alongside the previous sample from the same donor, and a "standard" antiserum. The results of some such comparisons are listed in Table 12 , and illustrate the value of such information to the clinician.

The results suggest that:-

(1) Treatment with corticosteriods (prednisolone or dexamethasone) does not diminish the sensitizing potency of the serum.

(2) A course of injections of antigen leading to clinical improvement (hyposensitization) diminished the potency of the whole serum (one case only). Further studies are desirable (see discussion).

(3) There is no obvious seasonal variation in the potency of the serum which might be expected from renewed exposure to antigen.

PREPARATION OF ANTIGEN

In early studies "Pollaccine" a commercial extract of Cocksfoot and Timothy pollen (Beecham Research Labs.,) was used as antigen, after checking each batch to show that no significant amount of histamine-releasing substance was present. Later it was desirable to exclude all non-allergic histamine release and to avoid the small

amount of phenol used as a preservative in 'Pollaccine'; furthermore studies on the more important antigens and the labelling of antigen with ^{131}I were envisaged. Extracts were then made directly from the pollen and fractionated by ammonium sulphate as described by Malley, Reed and Lietze, 1962.

Cocksfoot pollen (Dactylus glomerata) kindly provided by the Bencard Allergy Unit, was defatted by percolation with anhydrous ether in a column at 4°C, dried in air, and stored at -20°C. until required for use. 10 grams of dried defatted cocksfoot pollen were suspended in 100 ml of 0.127M sodium phosphate buffer, pH 10.4, containing 10% glucose. The suspension was continually agitated by magnetic stirring for 24 hours at 4°C., the residue was partially removed by centrifugation at 4°C for 15 minutes at 2000g and finally the extract was obtained by filtering the suspension through a starch cake (Connaught Medical Research Laboratories) 1.5 cm., thick, on a sintered glass funnel (porosity 2), using gentle negative pressure. The product was called "DAE" (aqueous extract of defatted pollen).

28g ammonium sulphate was added to 100 ml of the 10% extract of cocksfoot pollen (DAE) and the pH adjusted to 10.4. It was left overnight at 4°C, the precipitate (0 - 48% saturated ammonium sulphate fraction) was collected by centrifugation at 2000g for 15 minutes at 4°C. A further 5g of ammonium sulphate per 100 ml was added to the supernatant and the resulting precipitate (49 - 55% saturated ammonium sulphate fraction) was collected by centrifugation at 2000g for 20 minutes at 4°C.

Each precipitate was dissolved in sodium phosphate buffer pH 10.4 and reprecipitated by ammonium sulphate. This was repeated 3 times, and the salt was then removed from the protein by gel filtration on G25 Sephedex in 0.154M NaCl in a column 1 cm diameter x 30 cm run at 4°C. The protein solution was concentrated in Visking tubing using Biodryex.

When the antigenic potency of each fraction was evaluated, the samples were diluted to 100 mls for comparison with the crude extract.

TABLE 13.

Comparison of different preparations of pollen antigens.

All samples of antigen contained an extract of the same amount of pollen in 1 ml; in experiments (i) and (ii) the concentration was equivalent to 1 g pollen in 20,000 mls, in (iii) it was equivalent to 1 g in 10,000 mls. Part (a) of each experiment uses tissue soaked in Tyrode solution (T) only, part (b) uses tissue sensitized by soaking in diluted reaginic serum.
The serum used in (i) and (ii) was SS 40, that used in (iii) was No. 52.
Key to antigen samples:-

Poll. = "Pollaccine" (Timothy and Cocksfoot)
DAE = total aqueous extract of Cocksfoot pollen
FI = precipitate 0 to 48% sat. salt
FII = precipitate 49 to 55% sat. salt.

Treatment	µg/g Tissue	% Hist. release of Total	as %
(i) (a) T + T	7.47	9.9	100
T + Poll.		10.7	120
T + DAE		8.9	89.8
T + FI		8.1	91.8
T + FII		8.8	88.8
(b) Ab. + Poll.		25.3	284
Ab. + DAE		32.1	360
Ab. + FI		29.1	327
Ab. + FII		32.1	360
(ii)			
(a) T + T	17.9	6.4	100
T + Poll.		7.2	112.5
T + DAE		5.9	92.2
T + FII		7.2	112.5
(b) Ab. + Poll.		54.4	850
Ab. + DAE		52.1	814
Ab. + FII		51.9	810.9
(iii)			
(a) T + T	12.1	5	100
T + Poll.		5.9	118
T + DAE		3.5	70
T + FII		3.8	76
(b) Ab. + Poll.		10	200
Ab. + DAE		21.9	438
Ab. + FII		14.1	282

Tables (ii) and (iii) show that the total aqueous extract DAE was generally superior to and never inferior to Pollaccine. Fractional precipitation by ammonium sulphate at 48% saturation (FI) and again at 55% saturation (FII) gave two products containing antigens FI contained 1.2mg/ml of protein, and seemed to be slightly less active than FII which contained only 0.68mg protein per ml and no colouring matter. Since Malley et al had stated that FI contained antigens to haemaglutinins, attention was concentrated on FII. In tests to compare the effective levels of antigen by serial dilution (Table 14) it was shown that Pollaccine did not contain all the antigens necessary for adequate challenge, since in 14 (B) DAE diluted 100 fold more gave a greater release of histamine. With a different antiserum 14 (A) no difference is seen. Over a different range of comparison 14 (B) the total extract DAE is more effective than FII by a factor of about 100:

The use of DAE at a concentration equivalent to 1 g pollen in 10 litres seems to provide a margin of safety against variations in the antiserum. Table 13 shows that the spontaneous release of histamine is low with this extract.

TABLE 14

Comparison of the potency of preparations of pollen antigens and the establishment of suitable concentrations for routine use.

Two experiments, (A) using serum SS 40, and (B) using serum 52, each on different specimens of tissue. Dilutions of antigen show the volume of solution (mls) containing the equivalent of 1 g pollen. Key as on p ____.

Antigen	dilutions	Anaphylactic histamine releases (%)	Tissue hist. µg/g
(A) Poll.	1 = 2,000	44.7, 49.7	
	1 = 10,000	41.4, 49.7	
DAE	1 = 2,000	41.5, 51.0	
	1 = 10,000	41.5, 49.9	17.9
	1 = 50,000	54.4, 53.9	
FII	1 = 2,000	44.4, 44	
	1 = 10,000	46.7, 48.8	
	1 = 50,000	46.7, 49.0	
(B) Poll.	1 = 10,000	2.6, 5.7	
	1 = 100,000	0.6, 1.3	
	1 = 1000,000	1.9, 0.1	
DAE	1 = 10,000	20.6, 16.2	12.1
	1 = 100,000	9.5, 19.5	
	1 = 1000,000	9.9, 10.9	
FII	1 = 10,000	10.6, 10.0	
	1 = 100,000	6.0, 6.5	
	1 = 1000,000	4.8, 2.9	

ROUTINE PROCEDURE FOR THE SENSITIZATION AND CHALLENGE OF HUMAN LUNG

Human lung samples were collected within 30 minutes after the operation, in oxygenated Tyrode's solution. The pleura was then removed from the tissue with sharp scissors, and the underlying tissue was then cut into slices of about 5 mm thickness, avoiding obvious bronchioles. During this operation the tissue, and the instruments etc., were all immersed in Tyrode solution. The slices were placed on two thicknesses of Whatmann No. 1 filter paper and cut into rods approximately 0.8mm square, using a mechanical tissue chopper (McIlwain and Buddle 1953). The chopped tissue was transferred with a pair of smooth forceps in a beaker containing 300-400ml Tyrode solution. Finally the chopped tissue was stirred for a few minutes, and the excess fluid was sucked off by gentle negative pressure through a filter device made of perspex tube and silk mesh. This washing process was repeated three to five times as required and finally the supernatant was completely drained and the tissue divided into uniform samples of 100 mg (nominal wet weight) by a method based on that of Mongar and Schild (1953). The tissue samples were suspended

in 1 ml Tyrode solution in polystyrene tubes 7.5 x 2.5cm fitted with caps, and 1 ml of the sensitizing serum or antibody solution added. The tubes were gently rocked for about 18 hours at 17°C. At the end of the uptake period the tissue was drained and washed twice with Tyrode solution. It was then warmed in 1 ml Tyrode solution for 5 minutes and 1 ml of double strength antigen solution at 37°C was added. After incubation at 37°C with continuous rocking for 15 minutes, the supernate was sucked off from the tissue by the method of Mongar and Schild (1953), and heated in a boiling water bath for 5 minutes. Tissue histamine was obtained by transferring the tissue to 5 ml Tyrode and boiling it in a similar way for 5 minutes. All the samples were kept frozen until assayed.

BIOASSAY METHODS

Histamine

This was performed on a piece of terminal ileum of guinea-pig (250-300 g bodyweight), in Tyrode solution containing atropine 10^{-7} g/ml. The unknown solution contained substances other than histamine which may interfere with the assay, but these complications

Fig. 11 : Assay of supernatent fluid from tissue undergoing Ag-Ab reaction for histamine.

Unlabelled contractions of the guinea-pig ileum were produced by histamine acid phosphate equivalent to 2ng and 3ng base per ml bath fluid respectively.

Samples X, Y and Z from 3 samples of sensitized tissue.

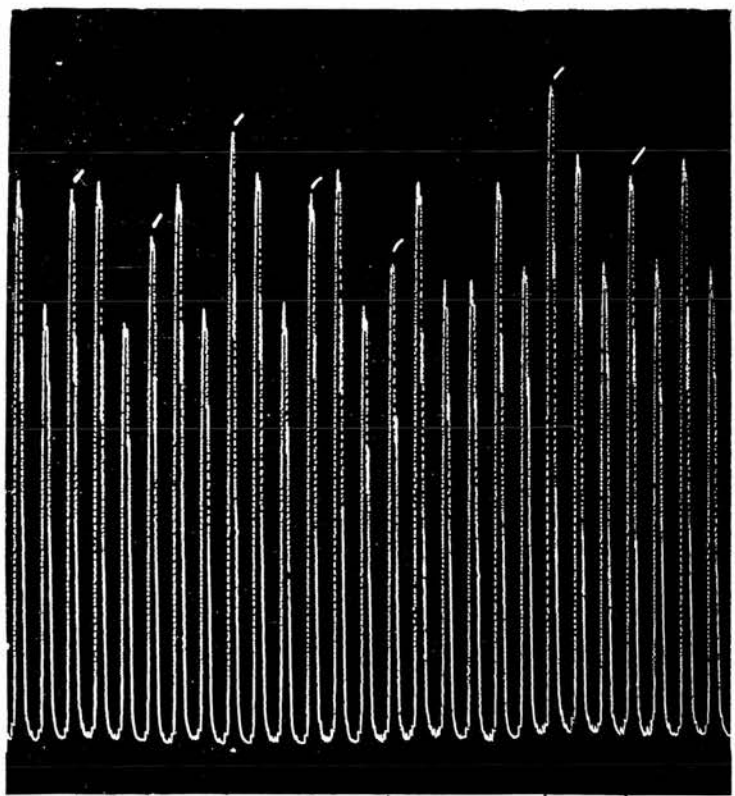
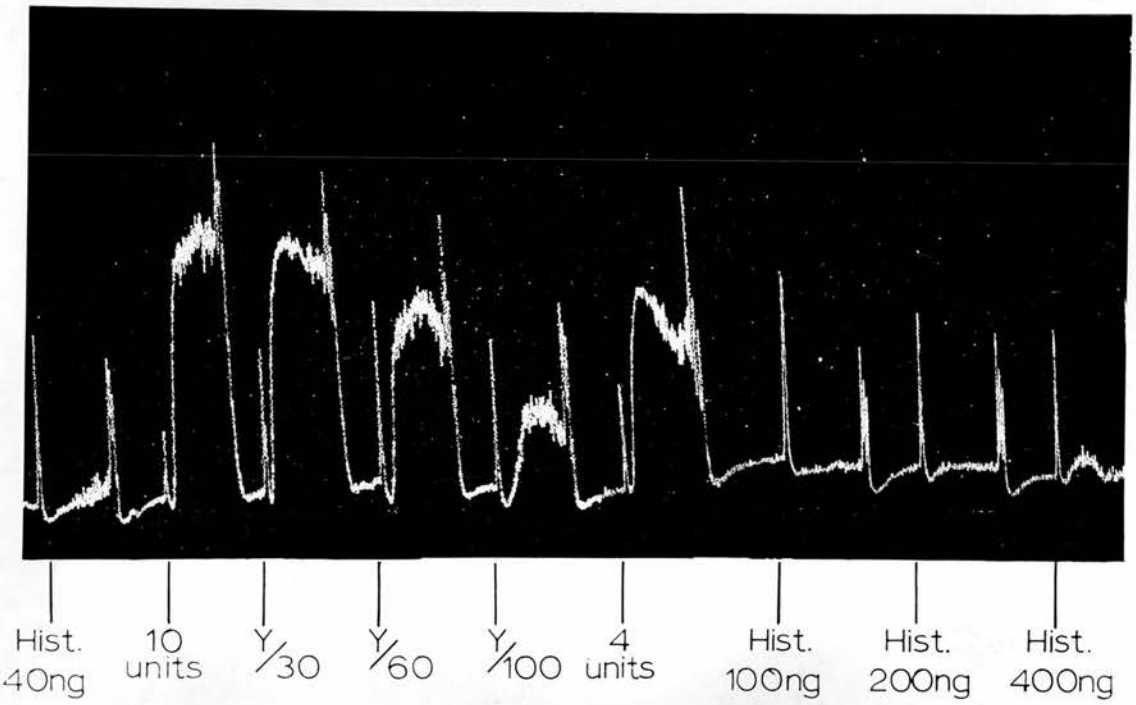

X 80
X 100
Y 80
Y 150
Y 200
Z 80
Z 150

Fig 12 : Test of the supernatant fluid from human lung tissue undergoing Ag-Ab reaction, to show presence of SRS-A.

The histamine equivalence of sample Y was 500ng/ml. Contractions of the guinea-pig ileum recorded in the presence of atropine 3×10^{-7}M and mepyramine 10^{-6}M.


Hist. 40ng
10 units
Y/30
Y/60
Y/100
4 units
Hist. 100ng
Hist. 200ng
Hist. 400ng

were largely avoided by working at high dilutions. Tissues which would not permit assay at less than 5ng/ml were excluded: the usual standard doses were 2 and 3 ng/ml (histamine base). Responses of each unknown were bracketed in between the responses to two standard doses of histamine and the results were calculated by interpolation.

The amount of histamine released is expressed as a percentage of the total histamine in the lung tissue. A typical assay is shown in fig. 11 .

Slow reaching substance (SRS-A)

The biological assay was usually performed on the same ileum as was used for the histamine assay, after mepyramine 10^{-6}M had been added to the Tyrode solution, and a period of 15 minutes allowed for the antagonist to produce a full effect. The response to SRS-A present in the supernatent was compared with the contractions produced by doses of a laboratory standard of crude SRS-A.

Fig. 12 is a record of a bioassay of SRS-A from human lung.

STUDIES ON THE PROCESS OF SENSITIZATION

All the antisera tested came from patients showing strong skin reactions, but only about 1 in 8 was good enough to be used routinely for passive sensitization of lung. There are two obvious explanations. The antigen used for skin tests was mixed grass pollen, whereas only Cocksfoot pollen was used as antigen on the lung (or Cocksfoot and Timothy in "Pollaccine"). The other possibilities concern the nature of the antibody globulins present or even the whole pattern of proteins in the serum. The skin test cannot be regarded as a measure of reaginic antibody, because γ_2 globulin could also take part, as it does in the guinea-pig where human reagins are without effect, (Augustin, and co-workers, 1966.)

Passive transfer (P-k) test would selectively show the presence of reagins, but tests in the antibody-producing (i.e. sensitive) subject are not selective. If other anti-pollen antibodies were present, they would compete for the antigen, but would be unlikely to totally abolish the anaphylactic release of histamine due to the

TABLE 15

The effect of mixing active atopic serum with inactive serum from another pollen-sensitive subject, on its ability to produce passive sensitization.

The reference serum (100) was used in a dilution of 1 in 40 and the poor sera were added in 1:20 dilution. The standard procedure was used for sensitization and challenge.

Treatment	Anaphylactic histamine release %
SS 100	10.4, 11.3
" + SS 121	10.4, 13.4
" + SS 145	9.2, 13.4, 7.9

Poor serum denotes a serum sample from an asthmatic patient, with a strong skin test against mixed grass antigen, but which failed to produce any sensitization of the human lung.

reaction between reagin and the antigen; alternatively if there was competition between different protein classes for uptake on the tissue, it is possible that uptake of reagin might be reduced to levels which were ineffective.

In the first type of experiment, serum samples from allergic subjects which had failed to sensitize lung were added to good samples during the period of uptake on the lung. It was thought that if no antibody was present, and there was no interference in uptake, then the sensitization should remain unchanged. If, however, the poor antiserum contained any factor which could inhibit uptake of reagin, or if it provided anti-pollen antibody which would attach to the tissue and have no effect other than uptake of antigen, their histamine release should be reduced.

Table 15 shows that there was no inhibition, although the ratio of anti-pollen reagin to other protein had been reduced by a factor of 3. Experiments with high dilutions of pollen fractions had suggested that some sera had very little antibody to pollen other than

TABLE 16

The comparison of fractions of γ globulins with the whole serum for ability to sensitize human lung.

The atopic serum was fractionated by Pevikon block electrophoresis or by filtration on Sephadex G 200 (see figures 13 and 14). The fractions were concentrated, and these were applied to the tissue in dilutions corresponding to 1:40 dilution of serum. NHS indicates addition of whole non-allergic serum 1 in 10. Results in duplicate.

Antibody Treatment	% Histamine release
SS_1 ($\frac{1}{40}$)	12.9 ± 1.2 (SD)
Pev = II	18.0
Seph= (A)	19.1
" = (B and C)	42.0
" = (A - E)	22.2
Pev = II + NHS	9.2
Seph= (A - E) + NHS	7.3
Controls	7.5

Fig 13 : Block electrophoresis on "Pevikon"

3mls dialysed human anti-pollen serum + marker Hb

Block 30cm x 10cm x 1cm. Buffer = barbitone pH8, 0.09M.

Sample into slot 3mm wide at cathodal end.

Applied voltage 350V - i.e. gradient 10-12V/cm gave current of 70mA.

Haemoglobin marker moved about 20cm in 17 hours.

Separation of γ_2 and γ_a from other globulins as shown, x = α and /or β globulin , other proteins have migrated anodally into the electrode bath. The types of globulin were identified by MIE or by specific precipitation (ouchterlony).

Separation cannot be inferred from the protein contents of 1cm strips of the block, which were estimated by absorption at 7,500 Å after Folin test, rabbit γ-globin was used as standard.

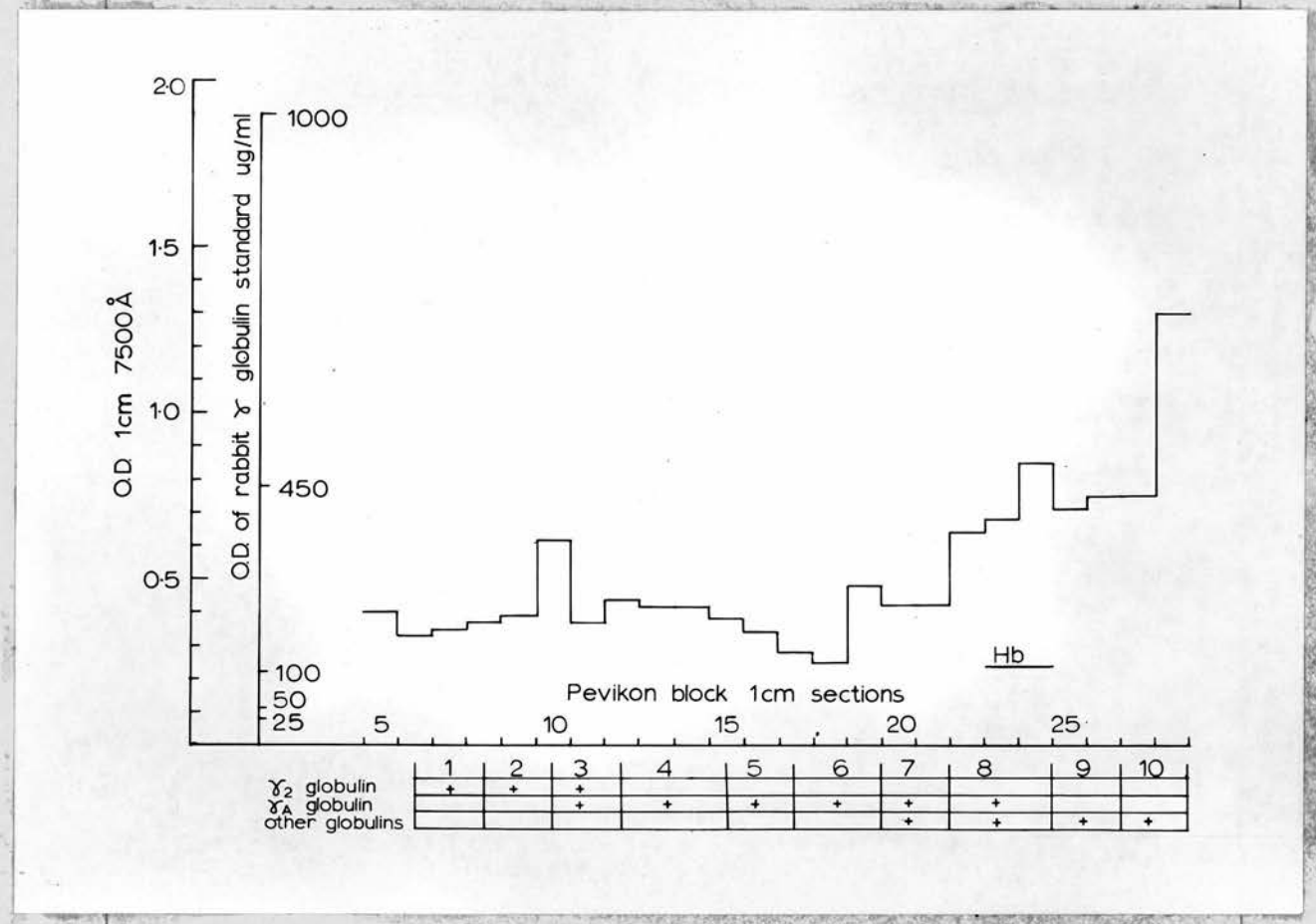

2·0
1·5
1·0
0·5
O.D. 1cm 7500Å
O.D. of rabbit γ globulin standard ug/ml
1000
450
100
50
25
Hb
Pevikon block 1cm sections
5
10
15
20
25
1
2
3
4
5
6
7
8
9
10
γ2 globulin
γA globulin
other globulins

Fig. 14 .- Separation of the proteins of human allergic serum by gel filtration.

Serum 4 ml adjusted to 1 M with NaCl. G 200 Sephadex in tris buffer pH 8, 0.1 M and NaCl 1 M.

Column 2.5 cm diameter x 80 cm: vol. 130 ml. Run at 17°C, 0.6 ml per min. 5 ml samples collected.

Protein estimated by OD at 280 mμ.

The 75 to 115 γ globulins are in peak II. The region containing reaginic activity is shown (grouped fractions A to D).

The sensitizing activity was tested on human lung - Table 16 .

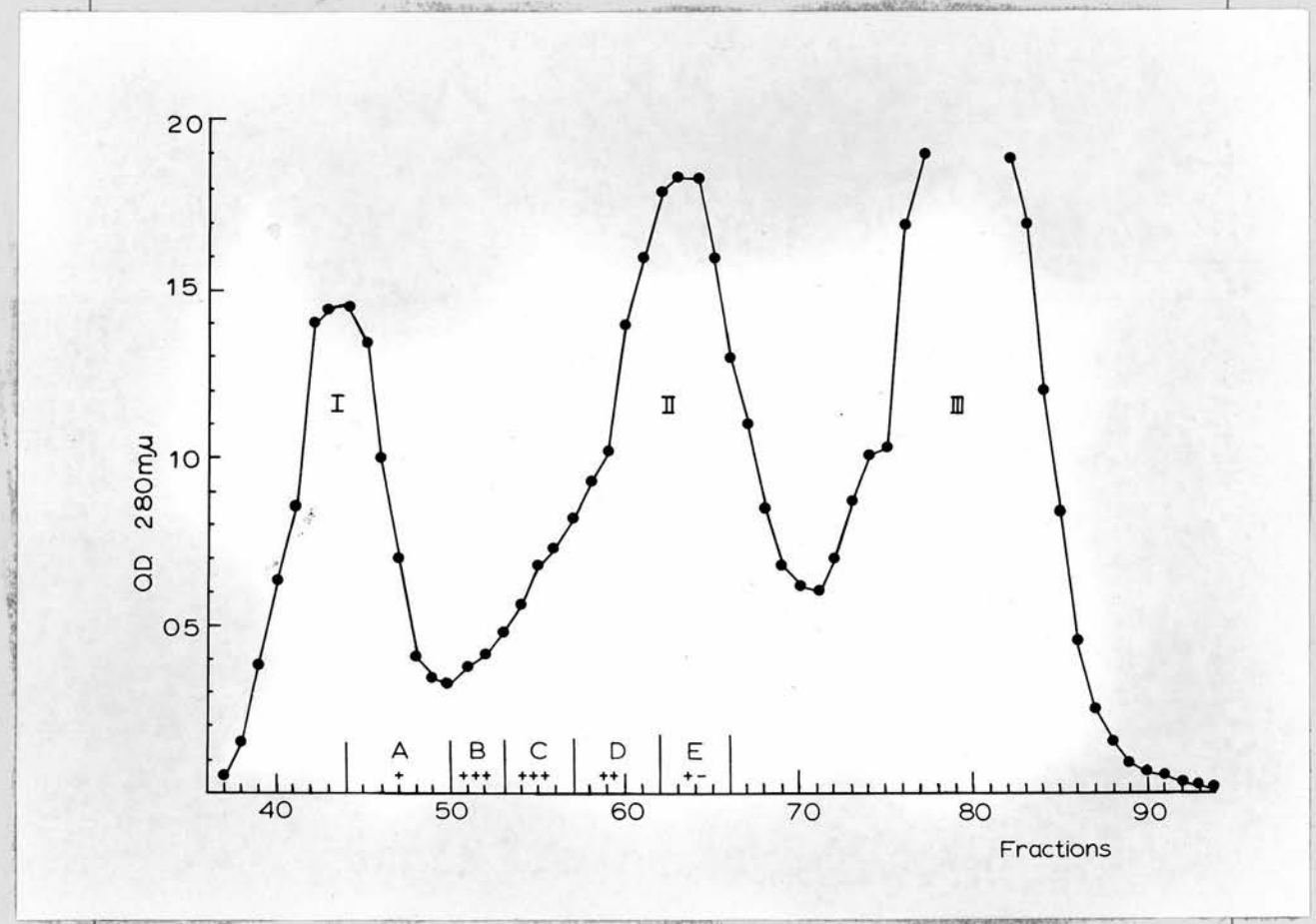

20
15
10
05
OD 280mμ
I
II
III
A
B
C
D
E
+
+++
+++
++
+-
40
50
60
70
80
90
Fractions

Fig: 15. Immuno electrophoresis of γ_1 globulin from pevikon block

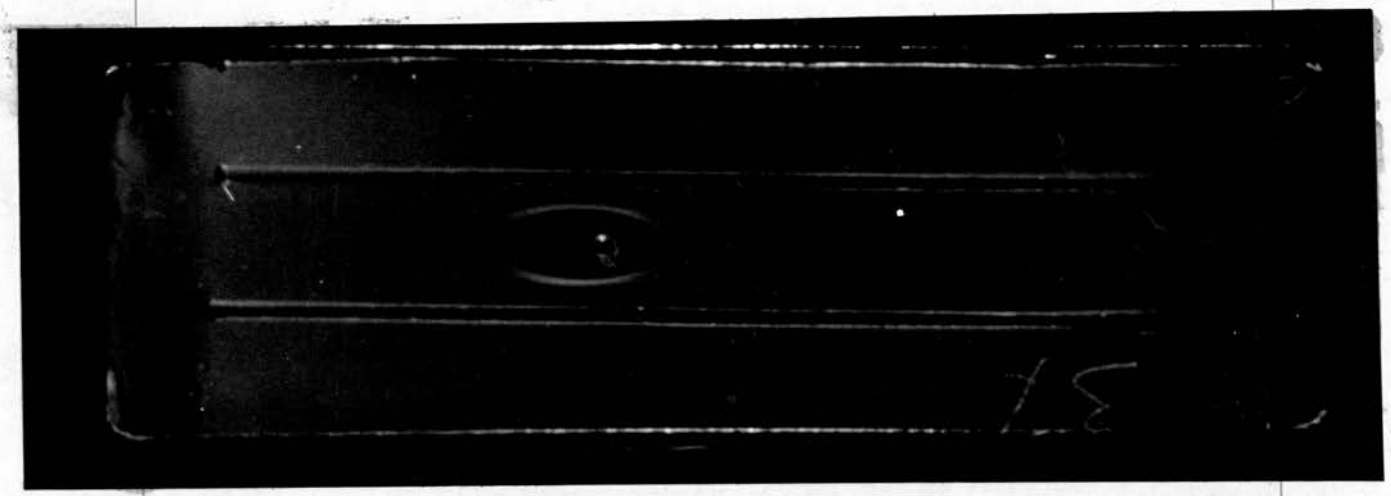
Top trough = anti γ_{1A} serum (HYL.)
Well = segment (5)
Bottom trough = anti whole human serum (HYL.)

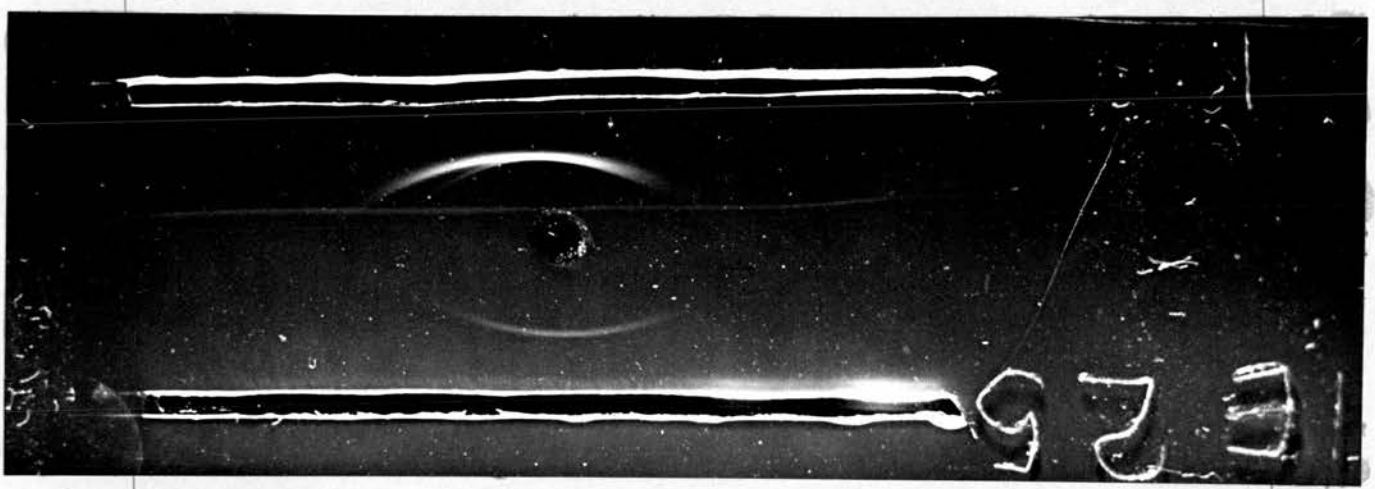
Fig: 16. Immuno electrophoresis of peak II (reaginic serum) on gel filtration

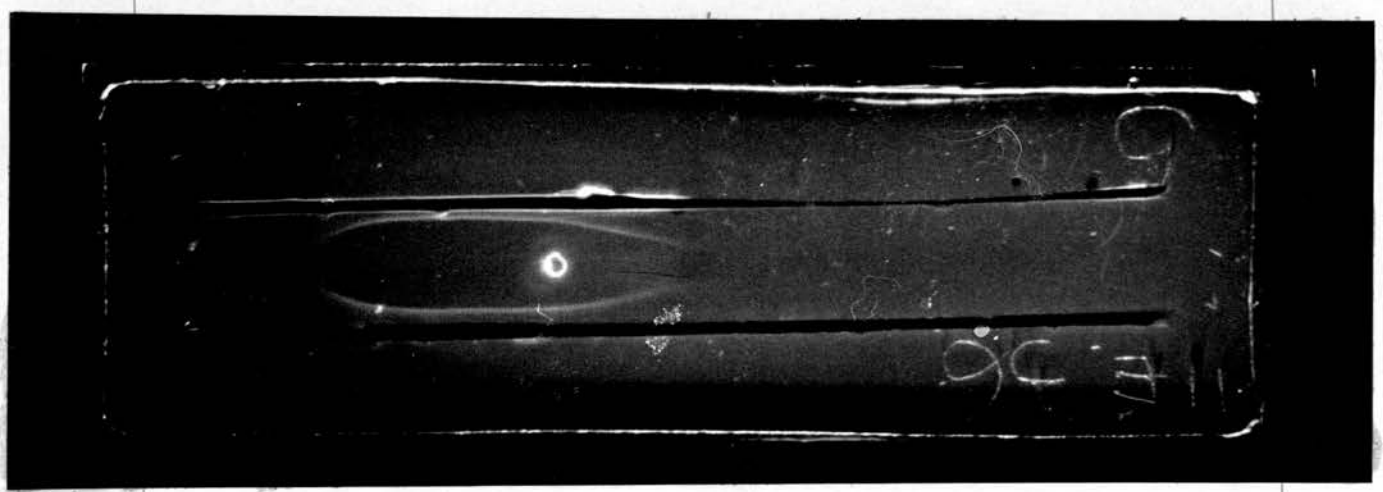
Top trough = anti whole human serum (HYL.)
Well = peak II (segment C)
Bottom trough = anti γ_{1A} (HYL.)

Fig: 17. Immuno electrophoresis of γ_G (normal human serum) on DEAE col.

Top trough = anti γ_G (Behringwerke)
Well = peak I (γ_G)
Bottom trough = anti whole human serum (HYL.)







the reagins, whereas others had antibody which remained in the tissue (not necessarily fixed) and reduced the antigen available for reaction with the reagin. The purification of reagins is not possible without the use of zinc or similar precipitants, but γ_2 globulin can be removed by comparatively gentle procedures, and reagin-rich fractions of γ globulin can be produced.

Experiments were performed in which the whole globulin was separated by electrophoresis of serum on Pevikon, and the part of the block corresponding to γ_A globulin separated (Muller-Eberhard 1960).
In table 16 this material is called Pev.II. The same anti-serum was also separated by gel-filtration on Sephadex G 200 and the region containing sensitizing globulins collected in 3 main fractions, identified by the protein-elution pattern and called A, B and C, D and E (Fig. 14) The sensitizing potency of these products was compared with the original whole serum, and the results are shown in Table 16 All the samples were diluted so that they corresponded to volume of the original serum,

and were thus directly comparable. Sample B and C was by far the most effective in producing sensitization, and it was noted that the tissue released a considerable amount of SRS-A as well as histamine when challenged. All the fractions were more effective than the whole serum although the Sephadex fractions must each have contained less reagin, since it was distributed through the fractions. It follows that there must be competition for uptake, especially if the effect of normal human serum (Table 16) is taken into account.

The nature of this competition can be inferred from a consideration of what proteins the different fractions do or do not contain:-

Pevikon II contains all the γ_A and no detectable γ_G

Seph A contains some γ_A and part of the γ_G and possibly a little of the γ_M

Seph B and C contains γ_A and part of the γ_G (see Fig. 14)

Seph D and E contains both γ_A and γ_G

It was concluded that there was some competition

between γ_A and other proteins, and that both γ_G and non pollen-antibody γ_A might be involved.

To throw more light on this matter, γ_G was separated on DEAE cellulose from the serum of a non-allergic subject who had spent most of his life in areas where Cocksfoot grass is not found. The chopped lung was soaked in a solution of this "normal γ_G globulin" containing 1 mg/ml, for 4 hours before exposure to the whole reaginic serum at 1 in 20 dilution (total protein about 3.6 mg/ml). In another test the γ_G was left in the solution when the whole serum was added. The results are given in Table 17 and show that pre-exposure to γ_G very greatly reduces the sensitization taking place during 18 hours. This long period when displacement failed to occur indicates very firm uptake of γ_2 on the tissue and confirms the concept of competition between γ_2 and reagins at least, for uptake sites on the tissue.

TABLE 17

The inhibitory effect of 'normal γ_G globulin on sensitization of human lung.

Tissue treatment	Anaphylactic hist. release	Tissue histamine	Inhibition
(1) Normal standard sensitization	31.6	18.9µg/g	
(2) Normal γ_G (1mg/ml) 4 hours, then drained and sensitizing antibody 1 in 20 applied 18 hours	19.5	17.5µg/g	38%
(3) Normal γ_G (1mg/ml) 4 hours, then sensitizing antibody 1 in 20 added	15.1	17.5µg/g	52%

SECTION 3

THE BIOCHEMICAL EVENTS LEADING TO HISTAMINE RELEASE FROM SENSITIZED LUNG

Introduction

The biochemical events finally resulting in the release of histamine during an anaphylactic reaction in vitro have been extensively studied in guinea-pig lung, but have not been amenable to study in human tissue. There is evidence to suggest that lung tissue from asthmatic patients behaves like the lung from sensitized guinea-pigs (Schild, Hawkins, Mongar and Herxheimer, 1951, Brocklehurst 1960). Studies on passive sensitization of human lung (section II) show that human lung tissue has much in common with guinea-pig lung, although the physico-chemical characteristics of the sensitizing antibody differ. It has to be assumed - until evidence is available - that passive sensitization of human lung in vitro is a true model of in vivo sensitization.

Several workers have produced in vitro inhibition of the anaphylactic reaction with metabolic inhibitors and by oxygen lack for example, Mongar and Schild (1957) in chopped guinea-pig lung and Chakravarty (1960) in

chopped guinea-pig and rat tissue. The report by Moussatche and Prouvost Danon (1957) that certain intermediates in the tricarboxylic acid cycle enhanced anaphylactic histamine release, supported further the concept that this needed metabolic energy. Austen and Brocklehurst (1961) have demonstrated inhibition of anaphylactic reaction in chopped guinea-pig lung by chymotrypsin substrates and inhibitors and also by fatty acids related to hexanoic (caproic) acid. These authors confirmed the enhancement of in vitro anaphylactic reactions by succinic acid, but showed that other Kreb's cycle intermediates such as malic acid and fumaric acid had little or no effect, whereas maleic acid was at least as active as succinic acid, to which it is structurally related although it is not part of any metabolic system. Only a limited time was available for systematic investigation on the human tissue, of those substances known to be effective on guinea-pig lung, so the programme of tests was restricted to those thought to be of greatest significance. The order of the tests roughly follows the historical sequence in guinea-pig work, namely:-

(1) temperature optimum, Ca^{++}, desensitization by heat

(2) metabolic inhibitors (SH)

(3) succinic and other acids

(4) chymotrypsin inhibitors and ester substrates.

Outline of routine procedure

The human lung samples were chopped and sensitized by soaking in antibody solution for 18 hours at 17°C, as described in Section II. The treatment of the tissue prior to challenge varied with the different experiments. Challenge employed a concentration of antigen equivalent to the extract of 1 mg pollen in 10 mls. (1 in 10,000), and always took place at 37°C for 15 minutes, with rocking.

Temperature

The optimum temperature during challenge was investigated briefly as mentioned in Section II (Table 7). It was shown that 37°C was a satisfactory temperature for histamine release, and that lower temperatures even for the first minutes of the period of challenge, depressed the reaction.

Desensitization by heating at 44°C for 20 minutes had been reported by Mongar and Schild (1957), and their

TABLE 18

The effect of moderate heating of sensitized tissue prior to challenge

Tissue histamine = 20.1µg/g

Treatment before challenge	Anaphylactic histamine release %
Normal Standard	54.9, 59.0, 59.0
" control	7.4, 7.0
Heated at 45°C, and challenged at 37°C	10.1, 10.4, 10.7

experiment was repeated. The sensitized human lung was placed in 2 ml Tyrode solution and warmed at 45°C in a rocking incubator. After 20 minutes the tissue was drained, washed with Tyrode solution at 37°C and incubated at 37°C in 1 ml Tyrode solution before adding the antigen.

The results given in Table (18), show that desensitization had occurred.

Dependence upon Ca^{++}

The need for Ca^{++} in the reactions leading to histamine release has been shown by Mongar and Schild (1958). Whether Mg^{++} may not also be required remains uncertain. In the experiment with human lung, passively sensitized tissue was exposed to Tyrode solution without Ca^{++} and containing EDTA (Na) 1mM pH 7.6 for 5 minutes prior to and during challenge. The histamine release was compared with that obtained in parallel experiments by the normal procedure.

Table (19) shows that removal of Ca^{++} totally abolished anaphylactic histamine release.

TABLE 19

The need for Ca^{++} in the anaphylactic reaction. The results are the mean values of triplicate tests.

Tissue histamine = 22.7μg/g

Conditions during challenge	Anaphylactic
Normal Tyrode	50.4
Ca^{++} free Tyrode + EDTA	0.8

Tests with substances known to enhance or diminish anaphylactic histamine release from guinea-pig lung.

The tissue was sensitized and washed, then the samples were incubated at 37°C for 4 minutes in 0.6 ml Tyrode solution. The substance under test was then added in 0.4 ml solution at 37°C, and after 1 minute the antigen was added in 1 ml Tyrode solution at 37°C. The inhibitor was thus in contact with the tissue for 1 minute. This time is arbitrary and was chosen for the following reasons. Austen and Brocklehurst (1961) used 3 mls bathing solution and added the inhibitor in 1 ml, 10 seconds before antigen. They reasoned that longer periods of contact might in some cases adversely affect the tissue even before the challenge, and reduce or enhance the release of histamine independently of the mechanisms of the anaphylactic reaction. The present work used smaller volumes of solution, since the sensitization and challenge procedure was originally modelled on that of Colquhoun and Brocklehurst, but the tissue was cut into larger pieces for reasons mentioned earlier. There is a lower limit to the volume in which the enhancer or inhibitor substance can be added: this is determined by the accuracy with which the addition can

be made, and the solubility of the substance. When 0.4 ml was added it was feared that cooling might occur during addition, and believed that 1 minute contact could not severely harm the tissue, but would give a better chance for the temperature to equilibrate and the substance to mix adequately in the small volume.

The substances were dissolved in a minimum amount of isotonic saline, with warming if necessary, then the pH was adjusted to 7.6-7.8 using minute pieces of phenol red indicator paper, and the solution diluted as necessary with Tyrode solution.

The results are summarized in Tables (20, & (21), where the substances are classified according to their prinicpal mode of action.

There is a striking similarity to the reported findings with guinea-pig lung. The most outstanding difference is failure to enhance the reaction by more than a token amount with even large amounts of succinic acid. Another difference is seen with caproic acid which failed to inhibit unless present in high concentrations which may cause cell damage, and will certainly bind appreciable amounts of calcium.

TABLE 20

Inhibition of anaphylactic histamine release from chopped and sensitized human lung by ester substrates or direct inhibitors of known enzymes

Key:-

Chym = Chymotrypsin
Tryp = trypsin
Tyr. = tyrosine
Trypto = tryptophane
lys = lysine
Arg = arginine
Ac = acetyl
Me = methyl
Et = ethyl
e = ester
A = acid

Values for 50% inhibition in guinea-pig lung taken from

Mongar and Schild (1957)
Austin and Brocklehurst (1961)
Edman, Mongar and Schild (1964)
Mota, Da Silva, and Fernandes (1960)

Type	Compound	Conc^{n}. mM	Inhibition %	Estimated Conc^{n}. for 50% inhibition in human lung	Estimated Conc^{n}. for 50% inhibition in guinea-pig lung
I Chym. Inhib.	Phenol	0.5	6		
	"	1.0	39		⩽1mM
	"	1.0	43	1mM	4mM
	Indole	0.1	9		
	"	0.5	9		
	"	1.0	80		
	"	1.0	52		
	"	3.5	93		
	"	5.0	100	1mM	⩽0.5mM
	Nicotinamide	5.0	55		
	"	5.0	49		
	"	5.0	71		⩽5mM
	"	20	88	5mM	10mM
	Phenyl acetic acid	5.0	18		
	"	10	6		
	"	10	15		
	"	10	20	⩾10mM	⩽5mM
	Indole acetic acid	2.5	16		
	"	10	30	>10mM	⩽2.5mM
	Indole propionic A^{-}	2.5	26		
	"	10	32	>10mM	⩽2.5mM

Table continued.

Table 20 continued

Type	Compound	Conc^{n}. mM	Inhibition %	Estimated Conc^{n}. for 50% inhibition in human lung	Estimated Conc^{n}. for 50% inhibition in guinea-pig lung
II Chym. Substn.	L-leucine Et.e.	10	89	5mM	⟨5mM
	L-Tyr. Et.e.	5	75	5mM	⟨5mM
	N-Ac. L-Tyr. Et.e.	4	58	4mM	⟨4mM
	N-Ac. L-phenylalan. Et.e.	1	25	2mM	⟨1mM
	L-Trypto. Et.e.	2	71	2mM	⟨2mM
	N-Ac.L-Trypto. Et.e.	1	34	2mM	⟨0.5mM
⊛	Glycine Et.e.	10	70		
	"	20	59	10mM	⟩20mM
III Tryps. Substr,	Benz.L-Arg. Me.e.	10	22.5	20mM	⟩20mM
	Benz.L.Arg. Et.e.	20	7% (increase)		⟩20mM
⊕	L-lys. Et.e.	20	100		
	"	2.5	0		
	"	10	85		
	"	20	100	7mM	⟩20mM

⊛ = control ester

⊕ = weak chymotrypsin substr.

Table continued.

Table 20 continued

Type	Compound	Conc^{n}. mM	Inhibition %	Estimated Conc^{n}. for 50% inhibition in human lung	Estimated Conc^{n}. for 50% inhibition in guinea-pig lung
S-H block	Iodoacetate	2	79	2mM	2a < 1mM
"	N-ethylmaleimide	1	75	1mM	1a 0.1mM
Fatty acids	Capric acid	1	14		0.8mM
	Caproic acid	20	10		
	"	20	30		
	"	20	33	40mM	5mM

TABLE 21

The effect of succinic acid on anaphylactic histamine release from chopped and sensitized human lung.

Normal release %	Release when succinate added %	Conc. succinate mM	Tissue histamine µg/g
21.9, 28.3	26.9, 31.9	0.5	6.5
21.2, 22.7	21.2, 24.6	0.5	12.1
19.9, 17.2	21.5, 18.2	0.5	15.3
16.1, 15.7	18.9, 17.9	5	5.5
54.4, 52.8, 53.6	62.1, 71.5, 67.3	5	15.2

The most complete correspondence between the reaction in the two species is the effect of "chymotrypsin inhibitors" irrespective of their role as enzyme blockers such as phenol and indole, or as substrates presumably competing for the normal substrate in the chain of events leading to histamine release. Typical examples are acetyl tyrosine ethyl ester and acetyl phenylalanine ethyl ester. A finding which differs in the two species, is the small effect of the aliphatic acid derivatives of phenol and indole in human tissue. This agrees with the observations of clinicians who have found phenylacetic acid to be ineffective in human asthma. It also underlines the generally accepted view that the enzyme involved in anaphylactic release of histamine has an overall similarity to chymotrypsin but is not identical with it.

In both tissues also, the characteristic substrates for trypsin were without effect, and only those which have some affinity for chymotrypsin showed a little activity.

As might have been predicted, those substances which inhibit or destroy SH enzymes were highly active.

DISCUSSION

General

The symptom complex exhibited during anaphylaxis varies from species to species and with the method of sensitization and challenge, but an increasing volume of evidence suggests that the main part of the symptom complex is caused by the release of autopharmacologic substances by enzyme action. Despite the variation in the anaphylactic symptoms, it seems probable that the several types of reaction whereby active substances are released are essentially the same in different species. The clinician concerned with hypersensitivity believes that his problems are unique toman, but is forced to base new treatment on the results of experiments in another species. The experimental scientist sees an increasing correspondence between man and the laboratory animals as more knowledge becomes available, and he recognizes more and more clearly the need to subdivide the reaction into its component sections within one individual as much as between one species to another. The possibility of using human tissue in experiments practically identical with those widely used in the

guinea-pig arose from the demonstration by several groups of workers, that γ_1 antibodies would passively sensitize guinea-pig lung, and that these antibodies had some of the well established properties of human reagins. It is obviously important for the clinician to have confidence in the work of the scientist and desirable for the scientist to feel that his study is likely to benefit mankind. The use of human tissue would go a long way towards both these ends.

Concurrently with the present work there have been related studies by Dr. Altounyan and co-workers at Fisons Laboratories, Holmes Chapel and by Dr.Augustin and co-workers at Liverpool University. The former group have been concerned with elucidating the mode of action of anti-allergic drugs, and have limited their studies on sensitization to the development of a practicable technique with one antiserum. The group at Liverpool have been interested in a method for the detection of reaginic antibody in human serum, as an adjunct to the lymphocyte agglutination test (Ridges and Augustin, 1964). This group has studied a wider range of tissues, including human bronchioles, uterus, and appendix,

but of necessity such studies lack detail. Reaginic sera have been fractionated to show that the sensitizing antibody is present in the γ_1 fraction, but uptake has been for 4 hours or less.

It is thus apparent that the three pieces of work differ in their approach to the subject, but they have each gained in some measure from the experience of the others.

The aims of the present work have been detailed on pages 22 - 24 to explain the first stages of the research programme. These aims were not achieved in full, and the use of a single type of antibody was abandoned in the face of unexpected difficulty. It is now necessary to assess how far the value of the research has suffered as a result of these modifications and from being less complete than originally envisaged.

At this point in the study it is possible to see that the basic technique of sensitization has wide and very interesting applications. It is necessary to make the technique as simple and reliable as possible and to foresee criticisms and shortcomings. This is likely to require further experiments on the dynamics of

sensitization, since the method has been developed to a stage where it could be routinely used with confidence, but may well not be the most efficient and effective way of achieving sensitization.

Penicillin antibody in saliva

The experiments described at the end of section I failed to give passive sensitization and had to be abandoned. In retrospect there may have been nothing amiss with either the antigen or antibody used, and failure might now be attributed to insufficient time for adsorption of antibody. This cause of failure was not seriously considered at the time, and was not clearly shown until systematic studies had been made with the whole serum/pollen system. Skin sensitizing serum reagins to penicillin, and haptenic antibodies are now said to give good P-K reactions, so it would be worthwhile to test such an established antigen antibody system on human lung, and if successful, attempt to return to the use of salivary γ_1 antibody. There are very great advantages in the purity of the haptenic antigen especially in the Yagi and Pressman procedure for the location on MIE of specific antibody in the

various types of globulin. It would be unwise therefore to forget the early attempts to purify salivary antibody and thus use a single class of immune globulins, and a well defined antigen.

The separation of reagins from serum still raises serious problems of purity, and also leaves some doubt that the proteins remain unchanged after the use of Zn^{++} in the differential precipitation. There are obvious difficulties in the use of saliva. One is the presence of amylase, which is stated to separate with the γ globulins (Rahman 1965) and in the present experiments (page 27) was found where γ_2 globulin would have been after fractionation of the "purified" saliva on DEAE cellulose. The peak of activity corresponding to the γ_1 globulin did not contain amylase as shown by starch gel electrophoresis (Fig. 4), and was substantially free of any proteins other than γ_1 globulins. The possibility of adverse effects from bacterial action might be excluded by the use of azide on collection, since the subsequent purification procedure needed to remove amylase will also remove azide from the γ_1 globulin. If bbck electrophoresis was needed in future, polyacrylamide would be preferable to starch, if amylase is likely to be present.

The possibility of improving the technique of passive sensitization

The great difference in the time needed to passively sensitize guinea-pig and human lung, might be explained by assuming that the reaginic sera contained very small amounts of antibody. Halpern and co-workers (1959) found that the rate of sensitization increased with temperature and that if the concentration of antibody was halved, the time for equal sensitization had to be increased by a factor of $\sqrt{2}$. Augustin et al (1966) have sensitized human lung in 4 hours at 37°C with very high concentrations of antibody serum, but the sera used in the present study did not sensitize the lung unless applied for a longer time, and this necessitated a reduced temperature, (Table 5, and fig. 7). According to Halpern et al the effects of these changes in time (x 9) and temperature (÷ 6) should practically cancel each other. The difference may thus be due to the use of extremely potent serum by Augustin et al.

It may be unrealistic to apply the ideas developed from studies with guinea-pig antibodies to the studies on reagins, and instead to consider known differences, such as very strong attachment to tissue, as shown by the very great persistance of reaginic antibody at the site of intradermal injection. P-K reactions are possible two weeks or longer after injection of the reagins, whereas the corresponding reaction with guinea-pig γ_1-antibody becomes attenuated after the fourth.

day and cannot be provoked after 6 or 7 days. There is thus a difference in persistence of homologous antibody in the skin of the two species, indicating a difference in firmness of adsorption in the face of competition from the circulating γ globulins. In the isolated lung, the concentration of applied antibody is only one twentieth (or less) of that with which the tissue was equilibrated in vivo. Until much of this fixed antibody is lost, very little of the applied antibody will be taken up, since it has been shown (albeit with other types of antibody globulin, Ovary and Benacerraf 1962, Brocklehurst and Colquhoun 1965, Feigen et al. 1962.) that the amount adsorbed is linearly related to the applied concentration. The long period needed for sensitization may thus be the result of slow loss of antibody already on the tissue. If this could be speeded up, sensitization might be both quicker and more complete. It would be interesting to see if changes in ionic strength of the bathing medium would alter both loss of globulins from the tissue and later uptake of reagins. The experiment suggested would be to soak the tissue in a large volume of "hypertonic Tyrode solution" containing

say 0.4M NaCl, and then add the reaginic antibody in slightly hypotonic "Tyrode" containing 0.1M NaCl. If successful this procedure would spread sensitization and possibly make the test more sensitive to antisera containing little reaginic antibody. An alternative method might be to use CO_2 to wash off the fixed antibody before attempting to sensitize the tissue, since Halpern et al (1959) have shown that "desenstization" by removal of antibody does occur in some tissues when exposed to 100% of CO_2, pH 5.8.

The consequences of using reaginic serum

Every sample of human serum is likely to have different characteristics, (a) in respect of the proportions of the different types of immune-globulin (to a given antigen) present, (b) in the range of antigens, even from the one source (e.g. cocksfoot pollen).

We have neither knowledge of nor any control over the manner of sensitization, and the possible intervention of adjuvants or of other antigens, and we have only a history of symptoms as a guide to the duration of exposure to antigen. There is no question that the use of whole human reaginic serum greatly complicates the

study of passive anaphylaxis at every stage. During sensitization of the tissue there will be competition between different types of immune globulin, as is clearly shown in tables (16) and (17). The removal of γ_2 globulin greatly enhances the sensitivity produced by the remaining antibody, presumably γ_1, and the addition of non-specific γ_2 reduces the sensitization. In whole serum, the ratio of anti-pollen reagins to other immune globulins will obviously determine the sensitizing potency of the serum. There is no evidence of any major change in the proportions of the main types of γ globulin, but those subclasses such as the reagins have never been accessible to quantitative measurement, and may well be raised in hypersensitivity. The difference in sensitizing potency of the whole sera is most likely to depend upon the actual level of specific antibody reagin, but since competition does exist it may be possible to convert 'moderate' sera to good ones by removal of γ_2 globulin. There may also be competition for antigen, especially if "blocking antibody" is present, (Cook et al 1935). Unless the ratio of other anti-pollen globulins to reagin is very high the wide range of antigen giving the same

anaphylactic yield of histamine, indicates that competition for antigen is unlikely to be important, and it could certainly be excluded by raising the doses of antigen used. Different antisera certainly have different antigen requirements, as shown in Table 14 : some show diminution of response with reduced concentrations of antigen, whilst others show no change. This is seen particularly with antigen fractions, and may occur because the antibody is directed to a major component of the pollen proteins in the one case, and a minor component in the other.

About 50 samples of serum from allergic patients have now been tested. Of these 7 or 8 gave good sensitization in dilutions of 1 in 20 to 1 in 80, and in all but fifteen sensitization was clearly detectable. The failure to detect cocksfoot reaginic antibody in 30% of the antisera is not surprising since the skin tests were made with 'Bencard' mixed grass pollen antigen prepared from 6 pollens. It may well be that the 30% came from cases sensitive to grasses other than cocksfoot, and for the same reason it would be futile to try to correlate the severity of the skin tests with the potency of the serum. The alternative explanation may be that the

skin test in the individual producing the antibody, is a composite reaction involving both reaginic and γ_1 antibody, or either alone. The passive sensitization of lung should parallel the P-K test, and both of these reactions will give much more information about the presence of reaginic antibody than can at present be expected from the clinical skin test in an actively sensitized subject.

It would be instructive to use immunochemical procedures to analyse at least semi-quantitatively the different kinds of antibodies to pollen, present in "good" and "poor" sensitizing serum. This could be done by the Yagi and Pressman technique which employs ^{131}I-labelled antigen. Different kinds of protein are unlikely to take up the ^{131}I-label to the same extent, and any lipids present will probably label heavily and fix rather non-specifically to antigen-antibody aggregates. Attempts were therefore made to purify cocksfoot antigens in the hope of finding one which was "dominant". Separation of the antigens was based on the differential precipitation procedure used for timothy pollen (Malley, Reed and Lietze 1962). This approach was deferred when it became clear that even simple fractionation of pollen

extract showed differences in the antisera, and the range of antigenic substances must be quite wide. Except in selected cases this would obscure any correlation between antibodies and sensitization.

Now that myeloma proteins of γ_E globulin have been reported there is the possibility of using antisera to such proteins to absorb the γ_1 fraction of anti-pollen reaginic antibody and test the identity of this protein with the sensitizing antibody. The identification of antigens is however an important practical matter, especially in such unbelievably crude products as 'house dust', which must surely be susceptible to improvement, particularly if these antigens are to be used clinically in courses of hyposensitization. A colleague is currently testing antigens of Aspergillus and Cladosporum to see if it is the hyphae or spores which are the main cause of allergy to these fungi. The method works well with both allergens. In cases of hypersensitivity where one antigen was dominant it might be possible to follow the changes in the pattern and quantity of antibodies during clinical hyposensitization to see what was desirable, and how best to achieve it.

Even if the study was limited to the separation of γ_1 and γ_2 antibody, it would be possible to show if any increase occurs in γ_1 as well as γ_2 antibody, and whether successful desensitization is due solely to competition for antigen by large amounts of γ_2 ("blocking") antibody. The reasons for failure to become hyposensitized should also become apparent, and the study of cases where clinical errors of judgment had occurred might show what to avoid. On points of current interest, it should be possible to evaluate objectively the virtues of depot doses of emulsified antigen, and to learn more about the antibodies and antigenic determinants involved in drug allergy.

In large systematic studies where much preparation is involved it would be wise to check that the tissue was satisfactory. It has been shown that tissue can be prepared and stored for 1 day at least, so that a pilot experiment could be run with the fresh tissue, and the main experiment delayed until the result was known. The actual size of an experiment should ideally be much larger than those reported here so that there is a large amount of direct comparison within it. This may be

conveniently arranged because of the long period of sensitization. Antibody may be added to batches of tissue samples at intervals of (say) 1 hour to permit time for the individual handling of the samples during the course of the next day.

The levels of sensitization have been quite high in most of the routine experiments, making it easy to show inhibition. Anaphylactic release of 30% of the tissue histamine, is usual with dilutions of 1 in 20 of a moderately potent reaginic serum or 1 in 40 of a good serum. This release is well below the maximum possible, since with 1 in 10 dilution of a very potent serum, 80% release was obtained, a value greater than has been reported for guinea-pig tissue. If a pool of anti-serum was being collected, it would be prudent to include only those sera which gave 25% histamine release at 1 in 20 dilution.

Inhibition and enhancement of histamine release

The substances tested are representative of the more important classes of agent shown to influence the anaphylactic release of histamine from guinea-pig lung. The work is conclusive in some instances but only preliminary in others. The unavoidable variability in the level of histamine release in different experiments makes assessment of the "concentration needed for 50% reduction" both difficult and rather pointless compared with the earlier work.

The correspondence between the two species of lung in respect of the direct involvement of a chymotrypsin-like enzyme is clear, and implies a similarity in the antibody involved and the enzymes available in the two tissues. The correspondence between the enzymes is not absolute, since phenylacetic acid was inactive in the human reaction. This is of relatively little importance beside the clear indication from the use of substrates, showing that the enzyme is an esterase active at sites adjacent to aromatic amino acids and not like trypsin.

The failure of succinic acid to produce any significant effect is surprising if metabolic processes were involved. One would expect that tissue kept at

room temperature for many hours would be in adverse metabolic condition and any enhancement would be even more marked than in guinea-pig tissue subjected to a much shorter period of unphysiological treatment. It may be that some other stage in tissue metabolism has been disturbed, and succinic acid levels do not overcome this bottleneck. Alternatively the antibody involved or the enzyme available in the tissue may be different in the two species. In any case, the role of succinate is so obscure that in the present state of knowledge this discrepancy has little significance.

Only salicylaldoxime has been used to show the possible involvement of the third component of complement, and several experiments were spoiled because some tissues (but not all!) lose histamine when in contact with salicylaldoxime. Very clear inhibition (50%) was found when 1mM salicylaldoxime was present. Some of the esters of aromatic amino acids which inhibit release from human lung are known to inhibit C'_3 (Basch 1965) in comparable concentrations. These include acetyl-L-tyrosine, acetyl-L-tryptophan, and their ethyl esters, and benzyl L arginine methyl ester. It would be reasonable to extend the tests

to (say) glycyl-L-lencyl-L-tyrosine, and glycyl-L-tryptophan to see how far the correspondence was maintained, although it is always assumed that complement is not activated or consumed in reactions with reagins.

Some of the inhibitory substances used increased the "spontaneous" release of histamine in the controls. When this occurred the experiment was ignored unless the reduction in histamine liberated by the anaphylactic reaction was greater than the amount released spontaneously in the control containing inhibitor.

Release of histamine attributed to a direct action of the inhibitor was seen with indole 5mM, L lysine ethyl ester 10mM, and salicylaldoxime 1mM. In all of these instances the effect could be avoided by the use of lower concentrations which still gave inhibition of anaphylaxis, or the effect was small enough to be allowed for, or was only seen in some experiments.

The spontaneous release of histamine from human lung into Tyrode solution is much greater than had been experienced previously with guinea-pig tissue. It varied greatly between different samples of tissue, and in a few, which were firm and looked healthy, the loss

was as little as 2%. After tissue had been stored for 24 hours at 4°C, the rate of loss was frequently very low, presumably because the easily freed histamine had been lost already.

The loss during the re-warming period was always high, yet the tissue must be at 37°C during challenge, and will usually be losing histamine at a relatively high rate at this time. Attempts to stabilize the tissue at 37°C before challenge were expected to reduce the control values for histamine release, and to improve sensitization at the same time. The reduction in sensitivity during this period is surprising and has not been explained. It may be that the tissue is in oxygen debt with subnormal metabolic energy reserve, and that the rise in temperature leads to greater metabolic activity and a rise in CO_2. This might then diminish the amount of antibody held on the tissue as shown by Halpern et al.

High control histamine release occurred in one experiment in the unsensitized tissue and antigen tubes, although the controls containing sensitized tissue and Tyrode solution were low. The obvious conclusion was that the patient must have been sensitive to this

system although this was not established. This experience underlines the need for caution when using human tissue.

The ability to passively sensitize human lung opens many new approaches to the study of human reagins. In the past the only valid test has been the P-K reaction, but there are dangers in this, and there are very severe limitations on any experiments designed to modify the reaction. This new method is particularly important at the present time when great advances in the separation and identification of new types of antibody are taking place. The studies reported here indicate the basic principles of handling human lung and of sensitization with reaginic antibody. They also show that human tissue has much in common with guinea-pig lung sensitized with γ_1 globulin. They should be regarded as preliminary investigations performed to assess the usefulness and practicability of the method, and as such show that the procedure has great possibilities. Some of these have been discussed, and in the light of experience possible modifications of the procedure have been suggested.

PURIFICATION OF PROTEINS

(1) Preparative electrophoresis

Preparative electrophoresis was performed using polyvinyl chloride - polyvinyl acetate co-polymer particles "Pevikon C-870".

After washing the particles with barbitone buffer (pH 8.6, 0.09M), a suspension in this buffer was poured into a perspex frame 30cm. x 10cm. x 1cm., lined with thin polythene sheet. The block was 6.5cm. above the level of the buffer in the electrode chambers. Connections were made with three thicknesses of lint. Usually 10ml of serum were concentrated to 3-4ml using Biodryex, and this sample was then dialysed against barbitone buffer (pH 8.6, 0.09M). After cooling the block in a cold room at 1°C the sample was applied by cutting a slot about 3mm wide, about 5cm from the cathode end of the block. The slot ended about 1cm from each edge of the frame and a short distance from the bottom. The material removed from the slot was blotted on a few thicknesses of Whatman No.1 filter papers and mixed with the serum to form a slurry which was poured slowly and gently back into the slot. After smoothing the surface with a

palette knife it was covered with polythene and electrophoresis was performed in the cold room using a potential gradient of 10-12 V $cm.^{-1}$ Electrophoresis was continued until the β-globulins approached the anode end. Haemoglobin is a convenient marker for the β-globulin region. At the end of the run the block was cut into 1cm wide sections each of which was suspended in 10ml of 0.154M saline and the eluate separated on a sintered glass filter leading to a container with a side arm which was attached to a water pump. The particles were resuspended and the process was repeated until 30ml of eluate had been collected. The eluates from each section were analysed for protein concentration by the Folin method and concentrated by pressure dialysis at 1°C, through Visking tubing. The position of the different types of γ-globulin was established by micro-immune electrophoresis or Ouchterlony diffusion. The concentrated eluates were sterilized by filtering through "Millipore" Cellulose acetate filters (EHWP 01300).

(2) DEAE cellulose chromatography

Columns were made from diethylaminoethyl (DEAE) cellulose. All columns and samples were equilibrated

with 0.01M pH 8.2 sodium phosphate buffer. For the preparation of γ_2-globulins about 7ml of equilibrated serum was applied to a column of 1.0cm diameter and 40 to 45cm height and elution carried out with the same buffer as was used to equilibrate the column.

To separate the total γ-globulins obtained by preparative electrophoresis the samples were concentrated to a volume of 3 to 5ml using Biodryex, and then dialysed against 0.01M pH 8.2 sodium phsophate. The concentrated samples were applied to columns 1.5cm diameter and 35 to 40cm height. After the first protein peak had been eluted, using 0.01M pH 8.2 sodium phosphate buffer a uniform gradient of rising ionic strength was started using a device of the sort described by Peterson and Sober (1959). The initial buffer was that used for equilibration of the sample and column, and the final buffer was the same with the addition of sodium chloride to a total concentration of 0.3M. Buffer was pumped from the gradient generator and the flow adjusted at about 40ml/hour. Fractions of about 10ml were collected and scanned for protein concentration by measuring their optical density at 280 mμ. Fractions were concentrated

by pressure dialysis at 1°C against 0.154 M saline.

(3) Gel Filtration

Sephadex G 200 was prepared and swollen by a method of Borsos and Rapp (1965). 20g of beads retained on a 200 mesh sieve were transferred to an Erlenmeyer flask containing about 700ml of 0.154M NaCl, and the suspension was boiled for 5 to 10 minutes. After cooling to room temperature, the slurry was poured into a chromatographic column 2.5 x 80cm whose sintered glass bottom was covered with a layer of Sephadex G25 about 0.5cm thick, and equilibrated with 0.1M Tris-HCl buffer, pH8, containing 1M NaCl. The serum sample (3-5ml) adjusted to 1M NaCl by the addition of solid NaCl, was applied to the column and filtration was performed with the equilibrating buffer. The flow rate was adjusted to about 0.6ml/minute and fractions (about 5ml) were collected (8 mins.). The optical density of each fraction was measured at a wave length of 280 mμ in the Beckman DB Spectrophotometer. Peaks I, II, and III (see Fig. 14) were concentrated at 4°C by pressure dialysis (1 atmosphere) against 0.154M saline, and sterilized by "Millipore" filters.

(4) Micro-immunoelectrophoresis (MIE)

Immuno electrophoresis was performed on microscope slides on a method described by Scheidegger (1955) using 1% Ionagar No.2 (oxoid) in veronal-acetate buffer (0.096M pH 8.6).

The slides were connected to the buffer chambers by Whatman 3MM paper and a current of 5mA/slide was applied in a humidified box for about 60 minutes. The results were photographed on Ilford FP3 film, using dark ground illumination, at various times after the addition of the antisera.

(5) Gel Double Diffusion

These were performed on microscope slides as described by Hartmann and Toilliez (1957) in a 1% agar gel as used for micro-immunoelectrophoresis. The peripheral wells had a capacity of 4µl and the central well had a capacity of 8µl. The results were photographed in the same way as MIE.

Preparation of precipitating Antisera

(a) Rabbits were immunised by injecting normal human serum in "Difco" complete Freund adjuvant (1 vol. + 1 vol).

Six doses of 0.1 ml emulsion were given initially (four intramuscularly and two subcutaneously), and after two or three weeks 5 doses of 0.1 ml serum diluted in 1 ml of 0.154 M saline were given by slow intravenous injection at two day intervals and the animals bled one week after the last dose.

(b) Miscellaneous Antisera:-

The following commercially available antisera were used:-

(1) Goat anti-human serum 3802 A1 (Hyland).

(2) Anti-human serum for incomplete Rh. antibody K5282 (Welcome)

(3) Goat anti-human γ_A 71-212 (Hyland)

(4) Rabbit anti γ_G globulin 905B (Behringwerke).

LIST OF MATERIALS

Cellulose acetate filters EHWP 01300, Millipore filter Corporation, Bedford, Massachusetts.

Visking tubing 18/32", supplied by H.M.C., 52 Gloucester Place, London, W.1.

Swiss Silk (standard mesh No. 15), John Staniar and Co., Sherborne Street, Manchester, 3.

Starch (hydrolysed), Connaught Medical Research Laboratories, University of Toronto, Canada.

Pevikon C.870 Vinyl Copolymer medium No. 2488, Shandon Scientific Co. Ltd., 65 Pound Lane, London, N.W.10.

DEAE Cellulose (7392), Kodak Ltd., Kirkby, Liverpool.

Sephadex G 200, G50 and G25, Pharmacia, Upsala, Sweden.

Ionagar No. 2, (L 12), Oxoid Ltd., London, S.E.1.

Biodryex supplied by Sigma, London.

Cocksfoot Pollen (1966 crop) and Pollaccine from Beecham Research Laboratories, Brentford, England.

Rabbit gamma globulins, Pentex Inc., Kankakee, Illinois.

Penicillin G, purchased locally.

HSA (No. 1701) Behringwerke AG., Germany.

Salts and solvents, (Analar) British Drug Houses, Poole, England.

All the inhibitors, esters, acids etc., were supplied by the Sigma London, 12 Lettice Street, London, S.W.6.

Difco complete Freund Adjuvant, Baird and Tatlock Ltd., 124 Great Ancoat Street, Manchester.

Goat anti-human serum 3802 A1, and anti-human γ_A 71-212 Hyland Laboratories, LosAngeles, California, U.S.A.

Rabbit anti-human γ_G globulin 905 B, from Behringwerke AG, Marburg-Lahn, Germany.

Anti-human serum K 5282, Burroughs, Wellcome, London.

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